THE USE OF END-TO-SIDE REPAIR OF PERIPHERAL NERVES FOR NEUROTIZATION AFTER LOSS OF NERVE TISSUE IN A LARGE ANIMAL MODEL

by

Sarah J.A. Kettle

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DECLARATION

I, Sarah Jane Alma Kettle, declare that this thesis has been composed totally by myself and that the work reported here is my own.

Any technical assistance has been acknowledged

This work has not previously been submitted for a degree at this or any other University.

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CHAPTER 3 – RESULTS

ABBREVIATIONS

An explanation of the terms used to label the different variables in the various tables and graphs presented here in this thesis, are shown here:

NEURO: neurotmesis and end-to-end epineurial suture group.

GRAFT: neurotmesis and autologous 1cm nerve graft group.

WRAP: neurotmesis and CRG material wrap group.

CONV: 'conventional repair' group. A combination of all 3 of the above median nerve repair groups.

MCONT: median nerve control group.

UCONT: ulnar nerve control group.

E to S: end-to-side neurorrhaphy group.

DE to S: double end-to-side neurorrhaphy group.

MyelinM: myelin thickness of the median nerve. For the experimental groups this was distal to the repair sites.

FibreM: Fibre diameter of the median nerve. For the experimental groups this was distal to the repair sites.

AxonM: axon diameter of the median nerve. For the experimental groups this was distal to the repair sites.

G-ratioM: G-ratio of the median nerve. For the experimental groups this was distal to the repair sites.

MyelinU: Myelin thickness of the ulnar nerves distal to the end-to-side neurorrhaphy sites.

FibreU: fibre diameter of the ulnar nerves distal to the end-to-side neurorrhaphy sites.

AxonU: axon diameter of the ulnar nerves distal to the end-to-side neurorrhaphy sites.

G-ratioU: G-ratio of the ulnar nerves distal to the end-to-side neurorrhaphy sites.

TSJ: jitter of the FCR or FCU muscles.

V M-M and V U-M: Maximum conduction velocity (CV_{max}) of the median nerve. For the conventional repair groups this was the CV_{max} across the repair and for the end-to-side group this was the CV_{max} from the donor ulnar nerve across the end-to-side neurorrhaphy to the attached median nerve. For these groups the values for these variables, V M-M and V U-M, were the same. The only differences in the values for these 2 variables are those for the double end-to-side group. V M-M represents the CV_{max} from the proximal median nerve end-to-side attachment across the portion of ulnar nerve used as a 'bridge' to the distal median nerve end-to-side attachment. V U-M represents the CV_{max} from the proximal donor ulnar nerve across the distal end-to-side neurorrhaphy to the attached median nerve.

F M-M: minimum latency (ms) value of the f-wave response from the normal median nerve.

F U-U: minimum latency (ms) value of the f-wave response from the normal ulnar nerve.

F M-U: minimum latency (ms) value of the f-wave response across the end-to- side neurorrhaphy site from the distal attached median nerve stump to the proximal donor ulnar nerve.

tPk: time to peak (ms) or maximum contraction of the FCR muscle.

Pk: the peak or maximum force (N) produced during a single twitch contraction of the FCR or FCU muscle in newtons.

Pk/wt: Force produced by a single muscle twitch (N) per unit weight of the FCR or FCU muscle (N).

tR/2: time (ms) to half peak amplitude of contraction. This was read from the twitch trace on the oscilloscope screen from a line drawn perpendicular to the *x*-axis through a point on the trace that represented half the maximum twitch of the muscle in Volts, as the muscle was fatiguing.

ATw: the area under the twitch contraction curve to a point that represents half of the peak force of the twitch contraction of the FCR or FCU muscles. Areas are measured in mNs and this figure is called the time tension integral.

TwTi: From this area, the time tension index (N) can be calculated by dividing the time tension index by the time to half peak of muscle contraction. This is an indirect measure of the work done by the muscle and is measured in newtons.

Tet: Peak tetanic force (N) produced by the muscle.

Tet/wt: Force (N) produced by a tetanic stimulus of the muscle per unit weight of it.

TW/TET: Ratio of twitch tension to tetanic tension (forces per unit weights of the muscles- N/N).

Mass: Mass (g) of the FCR or FCU muscle.

Tet/2: Time (ms) for the FCR or FCU muscles to fatigue to half their peak contraction for the tetanus, from the start of muscle contraction.

ATet: Area under the tetanic tension trace to half relaxation of the FCR or FCU muscle.

TeTi: Time tension index of the FCR or FCU muscle for the tetanic tension produced.

Bridge: The section of the donor ulnar nerve between the neurorrhaphy sites of the double end-to-side groups.

End-to-side groups: As a pleural term, this refers to the both the end-to-side group and the double end-to-side group.

Donor nerve: Refers to the ulnar nerve to which the end-to-side neurorrhaphy(ies) were made.

Recipient nerve: Refers to the distal attached median nerve stump through which, it was hoped, collateral sprouts from the donor nerve would grow.

FCR: Flexor carpi radialis muscle.

FCU: Flexor carpi ulnaris muscle.

ABSTRACT

End-to-side nerve repair is an experimental technique for repairing peripheral nerves when severe injury renders the proximal nerve stump not available for end-to-end repair or the amount of soft tissue loss is such that the gap between the two nerve stumps is too large for conventional nerve grafting techniques. These types of injuries are unfortunately becoming more common, generally as a result from motorcycle accidents.

This study uses a large animal model to compare two variations of end-to-side neurorraphy techniques with conventional clinically established methods of nerve repair to assess the feasibility of end-to-side suture as a technique for possible future clinical use.

All the experiments were randomized and the author performed all the surgery. The nerve repairs were assessed electrophysiologically and histologically and the muscles supplied by the repaired nerves were assessed physiologically.

There were no significant differences in the outcomes of nerve repair between different conventional techniques. End–to-side nerve repair did support nerve regneration but it was all or nothing. When innervation of the distal nerve stump and the recipient FCR muscle did occur, the functional outcomes were inferior to conventional techniques of nerve repair apart from the twitch and tetanic tensions of the FCR muscle.

Double end-to-side nerve repair consistently supported nerve regeneration but this repair was inferior to conventional techniques of nerve repair in all measures of outcome except twitch and tetanic muscle tensions. It is likely that regenerating axons used the epineurial and perineurial layers of the donor nerve segment between

the two neurorraphy sites as a conduit for axon growth as well as regenerating axons from collateral sprouts in the end-to-side pathway.

The function of the donor ulnar nerves in terms of conduction velocity, was compromised in the double end-to-side repair but not the end-to-side repair. Assessment of the donor FCU muscles in terms of the muscle physiological experiments also needs to be properly tested.

Further study is needed to assess the effects of placing the proximal neurorraphy site further away from the distal site in a double end-to-side neurorraphy on nerve regeneration. It is not clear from this work why some of the end-to-side neurroraphies supported nerve regeneration and some did not, especially in the light of the fact than none of the repairs had dehisced macroscopically.

Although end-to-side neurorraphy did support nerve regeneration with sometimes good return of muscle function, the use of this technique as a clinical tool at this time cannot be recommended.

CHAPTER I — INTRODUCTION

HYPOTHESIS

That the outcome, in terms of function and morphology after end-to-side repair of a nerve, is similar to that achieved using conventional methods.

OBJECTIVES

The effect of a peripheral nerve injury on the quality of life of a patient can be considerable both in terms of loss of function due to paralysis of a limb and scarring which either impairs function or causes psychological problems due to unsatisfactory cosmetic results. For many patients this will be because of impairment of movement, sensation and proprioception of the limb. However chronic severe pain and scarring from tissue loss also contribute to morbidity, which psychologically, can be severe especially in young patients. Most injuries are caused by direct sharp trauma, for example by glass or knives, but high-energy mechanisms such as road traffic accidents and high velocity missiles such as bullets and shrapnel cause an increasing number. The forces involved in a high velocity road traffic accident produce crushing, traction and shearing injuries of nerves. In penetrating missile injuries, not only is there local crushing and laceration, but indirect injury also occurs radial to the path of the missile in the form of a cavity, from kinetic energy-transfer from the missile to the surrounding tissues. Both these patterns of

injury are usually associated with substantial tissue loss. This makes repair of these nerves an extremely difficult problem.

The conventional way to repair a nerve is by direct end-to-end epineurial or perineurial (fascicular) suture; both ways seem to give the same results in terms of function (Tupper, Crick, & Matteck 1988). When suture is impossible without considerable mobilization and tension on the nerves, a graft is often interposed between the two ends. This is usually an autogenous cutaneous nerve.

When proximal tissue loss is extensive, grafting is often not feasible and other methods of reconstruction of the nerves must be considered. The technique of neurotization has been developed as a treatment for irreparable intradural lesions of the brachial plexus. This treatment involves reinnervation the distal stump of the injured nerve with the proximal stump of an intact nerve, which is sacrificed. Yeoman in 1961 used the ulnar nerve as a graft to connect intercostal nerves to the musculocutaneous nerve in a patient with a brachial plexus lesion. This resulted in some return of active flexion of the elbow. However this is not ideal as it destroys a healthy nerve and the muscles it innervates(Yeoman 1984).

End-to-side nerve suture for the repair of peripheral nerves and brachial plexus lesions after severe tissue loss in high-energy injuries is a technique that has recently attracted a lot of interest. It may reasonably provide an alternative to long nerve grafts in treating injuries where there is gross loss of soft tissue and contusion. If the technique is shown to allow nerve regeneration and the useful recovery of function of a paralysed muscle group it may prove superior to the neurotization technique.

The technique involves suturing the distal stump of the injured nerve side-on to a window made in the epineurium of an adjacent intact nerve. The hope is that axons (collateral sprouts) will sprout from the intact nerve and grow into the end of the attached injured nerve to reinnervate the muscles and skin sensation it supplied. There has been work produced on a rat model and more recently in baboons, but with varying results (Mennen 1999). The specific details of these will be discussed later. In this project end to side nerve repair is tested on the peripheral nerves of sheep as their size relates more closely to that of humans and is therefore a good experimental model to use.

ANATOMY OF THE NERVE CELL

A nerve cell or neuron consists of the cell body with its dendrites and its extension or axon and the terminal nerve branches. The cell body contains neuronal cytoplasm or axoplasm and this flows as a continuous stream through the axon moves from the cell body. The cell membrane is called the neurilemma and this membrane becomes that of the axon (axolemma).

The structure of the peripheral nerve tissue differs from that of central nerve tissue in that in the central nervous system the neurons are supported in a rich network of oligodendrocyte and astrocyte processes, with little extracellular space. Peripheral nerves are made up of fibres (Schwann cell units and axons) surrounded by a collagen matrix.

The transition from central to peripheral nervous system occurs in the rootlets of the spinal nerves. Fibres of the anterior roots have their cell bodies in the anterior

horn of the grey matter of the spinal cord and those of the posterior roots have their cell bodies in the posterior root ganglion (Glasby & Hems 1995). These neurons may be termed 'pseudo-unipolar' as they have a single axon and no other prolongations from the cell body. Each axon bifurcates into peripherally and centrally destined branches after leaving the cell body.

In the peripheral nervous system the axons are closely associated with Schwann cells or 'lemmocytes.' The larger axons are wrapped along their length by a series of Schwann cells. The 'nodes of Ranvier' represent the gaps between adjacent Schwann cells. Smaller nerve fibres are contained in bundles by similar columns of Schwann cells. The Schwann cell produces a lipid sheath called 'myelin' and it is thought this is laid down by the Schwann cell in spiral layers by its movement around the axon (Webster 1993). Myelin also contains glycoproteins.

The axon-myelin-sheath-Schwann cell arrangements (nerve fibres) are organized into bundles. In the nerve roots outside the spinal canal there is little endoneurial collagen in contrast to the abundant content in peripheral nerves. In the peripheral neuron the nerve fibre has three layers, the epi-, peri-, and endoneurium to support the bundles. The epineurium is, in effect, the prolongation of the dural sleeve of the nerve roots and is composed of longitudinally directed collagen fibres and fibroblasts. The perineurium, which sheathes the bundles, is composed of flattened cells alternating with layers of collagen. It provides a barrier to diffusion because the cells are joined by 'tight junctions'. The endoneurium supports the fibres themselves. It consists of longitudinal cells and collagen fibrils of a different size and composition from extracellular collagen found elsewhere, including the epineurium (Thomas 1963) (Gamble & Eames 1964). It has been estimated that 45% of nuclei seen in transverse

sections of nerves are those of fibroblasts and that 60% to 85% of the cross sectional area of the nerve is made up of connective tissue (Sunderland & Bradley 1949; Thomas 1963). The relative amounts of fascicular and epineurial connective tissue vary from level to level along the same nerve. This connective tissue component of the nerve trunk is an important resistor of compression injury to the nerve.

Sunderland mapped out the arrangement of the nerve fibre bundles along the course of nerve trunks and showed that the bundles branched, fused and changed in number such that no fasciculus ran an independent unaltered course along the entire length of a nerve (Sunderland 1968). This arrangement means that at the nerve root, the nerve fibres representing the distal branches are intermingled and widely distributed through the fasiculi of the nerve. On the other hand, the fibres of the proximal branches are concentrated in that sector of the nerve from which branching is about to happen. This fact may account for the poorer prognosis of repair for proximal nerve lesions compared to more distal lesions. This is probably because the nerve ends must be more accurately aligned during repair to give the sprouting axons a good chance to grow down and meet their original, but now empty, endoneurial tubes. These tubes guide growing axons towards their correct motor end plate and correct muscle fibre or sensory organ. These growing axons will also be mixed with some of those that will end up in distal branches.

At the more distal parts of the nerve, the fascicular plexuses 'sort out' the different systems of fibres until finally, the fibres for a particular branch are arranged into their own fasiculus, or group of fasiculi, which leaves the nerve as a definitive branch.

Sunderland suggested in 1965 that there was some degree of topographical separation of nerve fibres according to their function over large lengths of the nerve trunk (Sunderland 1968). This statement was followed up by microneurographic studies, which confirmed Sunderland's ideas (Ochoa & Torebjork 1983; Schady et al. 1983). There is specific organization of sensory and motor fibres in the median nerve in the arm and this separation allows techniques of nerve transfer to be feasible. Oberlin transferred 10% of the functioning ulnar to the motor nerve of the biceps in four patients with C5-C6 root avulsion after brachial plexus injury. He found no significant impairment of hand function and also that stimulating individual bundles of nerve fibres allowed separation of those innervating flexor muscles of the wrist and fingers from those innervating small muscles of the hand (Oberlin, Beal, & Bhatia 1995).

NERVE INJURY

Severity of injuries to a nerve range from transient ischaemia, the mildest form of damage, where there is transient failure of conduction to complete severance, in which there is complete loss of function. The latter injury is unfortunately the most commonly seen and carries the worst prognosis.

Injury to nerves can be caused in several ways. Broken glass or knives producing complete or incomplete clean severance of the nerve is the most common mechanism of injury seen in Accident and Emergency departments. Traction injuries are often caused by road traffic accidents, especially those involving motorcycle riders. Traction causes stretching, which can result in a range of severity of injury to

the nerve from separation of axons which leaves the rest of the nerve elements intact, to complete and ragged separation of the nerve ends in severe cases. Heavy, blunt trauma can cause neurapraxia an axonotmesis or a partial severance injury. It may be associated with fractures, pressure of the bone ends on the nerve or its entrapment in the fracture itself.

Classification of nerve injuries

The violence that injures nerves can be extremely variable and is completely random in its effects on the nerve's functions. The consequences of nerve injury can only be predicted with absolute certainty if it is clear that the injury has been complete severance of the nerve, or has brought about total destruction of its elements. Most patterns of violence especially penetrating wounds to nerves cause mixed lesions. This can make classification and therefore subsequent decisions for management a difficult task; however it is important to note that management always depends upon accurate classification of the injury.

There are two main systems of classification that have been developed for nerve injuries and are widely accepted. They are those of Seddon from 1942 (Seddon 1942) and of Sunderland (Sunderland 1951). Seddon's is a functional classification relating the anatomical injury to the symptoms it causes. Sunderland detailed further types of nerve injury intermediate to Seddon's axonotmesis and neurotmesis groups but these are purely anatomical which make them difficult to apply when observing the nerve injury microscopically at operation.

Seddon's classification

Seddon stated that there were three categories of nerve injury:

1. **Neurapraxia** – The meaning of the word is nerve 'non-action'. It is defined as a benign disturbance of nerve conduction of relatively short duration compared with the following more serious types of nerve injury. Paralysis is mostly motor with little muscle wasting and no change in electrical excitability. There can be degrees of subjective sensory disturbance such as numbness and tingling but no autonomic disturbance. The injury to the nerve is such that no elements of the nerve fibre are separated, local demyelination does occur but no Wallerian degeneration. There is a local conduction block that can take anything from several minutes to several months to recover. Larger nerve fibres are more likely to be affected, this explains why there is mainly motor and proproceptive disturbance, as these are the largest and most thickly myelinated of peripheral nerve fibres. An example of mild neurapraxia would be that of 'Saturday night palsy' where the radial nerve is compressed against the posterior aspect of the humerus causing a wrist drop. The patient is typically intoxicated and falls asleep with his arm over the back of a chair. However, this type of mechanism of injury can actually cause the more severe injury of axonotmesis.

There has been much debate in recent years as to what a 'neurapraxia' injury really comprises. The vagueness of the etymology does not allow us to describe the injury to the nerve in the same way as with the more precise terms 'axonotmesis' and 'neurotmesis'.

2. **Axonotmesis** – this implies the axon is severed but the endoneurial tube stays intact. The injury here is more severe and Wallerian degeneration occurs. The results of regeneration are relatively good because the growing axons are directed by the intact endoneurium and therefore reach their original target organs.

3. **Neurotmesis** – is described as complete severance of the nerve trunk. Repair has to take place in order for recovery to occur. Wallerian degeneration always occurs and the added complication of axonal mismatch may occur when the axons are regenerating owing to disruption of the endoneurial tube.

Sunderland's classification

This classification system is based on the microscopic anatomy of the nerve and therefore involves more patterns of injury than Seddon's system allows for. There are two more groups between the axonotmesis and neurotmesis groups. It also includes a 'metabolic block' group, which represents ischaemic injury. This system allows for a more detailed picture of the probable extent of nerve damage and therefore a prediction of the kind of spontaneous recovery that may be expected.

1. **Metabolic block** – This represents loss of nerve conduction due to ischaemic changes of the nerve.
2. **First-degree injury (Type 1)** – Blockage of nerve conduction without axonal injury corresponding to neurapraxia.

3. **Second-degree injury (Type 2)** – Axonotmesis i.e. loss of continuity of the axon with preserved neurilemma and covering of the fascicle (i.e. the endo-, peri- and epineurium.) Wallerian degeneration occurs below the level of the lesion, so axonal continuity with the periphery is lost until regeneration occurs. Injuries of this type can occur as sequelae of chronic compression syndromes such as carpal tunnel syndrome and ulnar groove syndrome.

4. **Third-degree injury (Type 3)** – Loss of continuity of the axons and the endoneurial connective tissue with preserved perineurium and epineurium. The internal structure of the fascicule has lost continuity and is disorganized. Wallerian degeneration occurs with axon disintegration. This type of injury may occur after acute blunt trauma of the nerve, which leads to haemorrhage, an inflammatory reaction and then fibrosis.

5. **Fourth-degree injury (Type 4)** – Loss of continuity of the axon, the endoneurial tissue and the perineurium with the epineurium staying intact. The fascicular structure the nerve has been destroyed. This type of lesion occurs after severe blunt trauma or incomplete transection of a nerve.

6. **Fifth-degree injury (Type 5)** – complete severance with dehiscence of the nerve stumps corresponding to 'neurotmesis'. No useful regeneration of the nerve to provide useful function of the muscle the nerve innervates, will occur if the ends of the nerve are not brought together or interposed with a graft or other conduit for regenerating axons (Sunderland 1978).

It was suggested in 1988 that a partially divided nerve that has regenerated by forming a 'neuroma-in-continuity' should be included in Sunderland's classification.

This injury can combine all five of Sunderland's degrees of injury and constitutes the most challenging in terms of management (Dellon & Mackinnon 1988). However the Sunderland classification was never intended to consider 'after effects' of injury as this does not seem appropriate.

Problems with classification systems

As Dellon and Mackinnon suggested (above) injuries to the different structures of the nerve fibre can exist in different combinations making classification extremely difficult. Nerve fibres serve many functions, they differ in their size and coverings, differ in their vulnerability to injury, and are sometimes differently related in space to the line of injury. Taking into account the different mechanisms of injury that a nerve may suffer there are many variables that may affect what structures of the nerve and to what degree these structures are disrupted. The nerve fibre is so complex a structure that no classification can really portray the consequence of every grade of violence.

DEGENERATION AND REGENERATION

The idea that an end-to-side nerve repair could produce useful collateral sprouting from the donor nerve to the sutured recipient nerve for the latter nerve's reinnervation is based on our biological knowledge of nerve degeneration and regeneration.

Reactions of the nerve fibre to injury

Degeneration of a nerve can occur as a result of a variety of mechanisms of injury such as ischaemia, traction, pressure, stretching, distortion, cold, heat, severance, electric shock, ionizing radiation, infection, inflammation, tumour pressure and effects of systemic disease. These different mechanisms of injury can have very different microscopic effects on the nerves, which, in turn, can affect the potential for regeneration.

When a peripheral nerve suffers a Sunderland Type 1 injury (neurapraxia) e.g. single blunt blow or rapid displacement by a fast moving object, there is paralysis of the muscles that the injured nerve supplies for three to four days. In large myelinated fibres there is myelin swelling and the start of dissolution on day three. Small fibres appear to remain intact.

Wallerian degeneration

An axonotmesis injury causes division of the axons, myelin and Schwann cell sheaths (but not their basement membranes,) but the tubular endoneurial sheaths stay continuous throughout the injured zone. The axoplasm is free to flow down these intact endoneurial tubes from a particular fibre occupied by remnants of its distal segment.

Where the injury to the nerve is neurotmesis, the nerve trunk is completely severed. When either of these injuries occurs i.e. axonotmesis or neurotmesis, a cascade of chemical and cellular changes occurs at the site of the nerve transection. This is 'Wallerian' degeneration, after *Augustus Waller* who first described it in 1850

after he had cut the facial nerve in a cat (Waller 1850). This cascade of cellular and molecular events occurs at the distal extent of that nerve and shows characteristic morphological features. Survival of nerve fibres occurs only if they remain connected to the cell body.

The time-course of these events varies with fibre type, species, and age of the individual, temperature, and distance from the lesion. In the research performed by Lubinska, using the phrenic nerves of rats, fragmentation of the axon began at the site where the fibres were cut and progressed distally at a rate inversely proportional to the thickness and the internodal length of the specific fibre. For thin myelinated fibres the time before the onset of Wallerian degeneration from injury was 25 hours as compared to 45 hours for thick myelinated fibres. Thick myelinated fibres were usually of the motor variety. Speed of distal progression of degeneration was approximately 250mm per day for thin fibres and 46mm per day for thick fibres (Lubinska 1977).

The first phase of Wallerian degeneration occurs one to four days after severance when fragmentation of the distal axon and mitochondrial swelling occur. The myelin sheath separates from the axon and breaks up and these degradation products accumulate in the form of myelin ovoids in the Schwann cells, which begin (2-4 days after severance) to multiply by mitotic division. They reach their maximum number around the 20^{th} day after nerve severance. Further degradation of the myelin sheath fragments is carried out by the Schwann cells themselves and also by invading macrophages. These are histiocytic in origin and degrade the myelin sheath to neural lipids. These appear seven days after severance of the nerve and are still present six to ten weeks later. Retrograde degeneration of the proximal segment

also occurs but only as far as the next proximal node of Ranvier (one myelin sheath segment.) The greater degree of violence to the nerve axon, the more intense the retrograde reaction. The degradation processes correspond to those occurring in the distal segment with axon dissolution and proliferation of Schwann cells with a tendency to grow distally (Cajal 1928). There is swelling in the cell body with chromatolysis also, at this time.

The second stage occurs ten to twenty one days after nerve severance. Degeneration has extended to the motor end-plate. The muscle fibres show the beginning of atrophy. The newly formed Schwann cells remain in the neurilemma of the dissolving nerve fibre, where they arrange themselves in longitudinal cell columns, called 'bands of Büngner'. These play an important part in nerve regenerative processes. From a growth 'bulb' formed at the demarcation line of the proximal stump, collateral axons begin to sprout. During this time also, there is increasing chromatolysis of the cell body. This cell reaction is the morphological expression of an adaptation of the neuronal metabolism to an increased rate of synthesis.

Changes in the nerve fibres are accompanied by changes in the endoneurial interstitial tissue and the nerve coverings as well. The endoneurium, which is initially dilated, begins to shrink after the digestion of the degradation products of the axon and myelin sheath. This process is slow if there is no regeneration. After a year the diameter of the fascicle can decrease by 50% or more. The reactive collagen content of the connective tissue increases in this phase, while the Schwann cells of the 'bands of Büngner' decrease in size.

Changes in the perineurial coverings have also been observed, mainly loosening of the cell layers. The epineurial tissue undergoes little change except for some oedematous swelling at the nerve end. This tissue should be excised at the time of repair.

Regeneration

In the event of an axonotmesis or neurotmesis injury to the nerve, where axons have been separated, the regenerative process is a lot more complicated and less likely to result in normal nerve function, than after the simple neurapraxia.

When a nerve has been transected, there is intense cellular activity at both the proximal and distal stumps. Myelin debris is cleared by macrophages preparing the distal stump for reception of the newly growing axon sprouts and Schwann cells proliferate from as early as the second or fourth day. Axons start to sprout with collateral sprouts arising from nodes of Ranvier at levels at which the axons are still intact, and terminal sprouts arising from the tip of the surviving axon (proximal stump).

It is thought that after division of the nerve, the absence of factors normally transported in a retrograde direction may be responsible for the initiation of axonal sprouting (Varon, Williams, & Gage 1987). The process begins in the axons of the proximal stump as this has remained in continuity with the cell body. At the most distal portions of the proximal stump, which have undergone retrograde degeneration, an expansion appears at the tip of the axon within a few days. This is the growth bulb or growth 'cone' and usually has several collateral axonal sprouts arising from it. It usually occurs in the region of the previous node. For regeneration it is of critical importance that the axonal sprouts reach the distal stump. This

process is facilitated by the fact that proliferating Schwann cells grow in large numbers from the distal stump and so 'meet' the axonal sprouts coming from the proximal stump (Thomas 1966).

There is always a gap between the faces of the proximal and distal nerve stumps, even if the nerve has been repaired end-to-end or with a graft. This gap is first filled with fibrin blood clot. Capillaries and fibroblasts grow into it from surrounding tissues and nerve ends (Thomas 1966). Advancing axonal sprouts accompany Schwann cells. When a nerve graft bridges the gap, this acts as a source of Schwann cells for the continuing sustenance of the axonal sprouts. In recent years Lundborg has shown in rats that in the laboratory axons can grow across a gap of 10mm (Lundborg 1988).

Proliferation of the Schwann cells at the site of injury occurs two to four days after initial severance of the nerve. Depending on the type of fibres, the number of Schwann cells increases ten to fifteen-fold in the process, reaching its maximal level at the 20^{th} day. Eventually excess Schwann cells degenerate to a level consistent with a more healthy nerve. In the distal stump the Schwann cells come to lie end to end in the empty endoneurial tubes forming 'bands of Büngner'. When the axonal sprouts reach the distal stump they enter these bands of Büngner and act to guide the sprouting regenerating axons down the endoneurial tubes. As long as the sprouting axons reach their original Schwann cell /endoneurial tube the regenerative capacity of the nerve is optimized and full function of the muscle or sensory organ could be restored.

During regeneration in a Sunderland type II injury to a nerve (axonotmesis), each regenerating axon, as it grows, is confined to the endoneurial tube that originally

contained it. This ensures that each surviving axon is inevitably directed back to the end-organ that it originally innervated.

In a Sunderland Type III lesion axon regeneration is complicated by intrafascicular fibrosis, which may block or delay some axons in their growth. Other axons may be diverted from their proper course. However, even those that may have successfully managed to pass the scar tissue between the nerve fibre ends may be constricted and therefore further growth and development is hindered. This disorderly axon growth may be so pronounced that the involved fasciculi form a fusiform swelling at this site. Loss of continuity of the nerve fibre or endoneurial sheath with its axons means that regenerating axons are not automatically directed towards their endoneurial tube that originally contained them. They may therefore be misdirected into foreign ones. The problem with this 'mis-wiring' is the fascicular composition is such that sensory and motor fibres and those representing functionally unrelated systems are intermingled and therefore do not have the same, functionally related destination. In a Type III lesion the perineurium is left intact so mis-wiring should be confined to specific fascicles. However, despite this, recovery after this lesion is often very poor.

When the injury is such that only a thin disorganized layer of epineurium is holding the nerve trunk together (Sunderland Type IV), regenerating axons are free to escape from perineurium and from the fasciculi. This segment of injured nerve is replaced by fibrous tissue, which halts axon growth. This injury requires excision of the damaged segment and a formal nerve repair, which may be in the form of a nerve graft if excision leaves a gap large enough to put the two nerve ends under tension.

In a repaired nerve, the first axon processes from the proximal stump were seen in the distal nerve at seven days after severance and suture (Barton 1962). Axons were seen to grow for short distances unsupported by other cells possibly attracted by soluble factors diffusing from the distal stump (Fawcett & Keynes 1990; Hall 1986; Lundborg et al. 1982a; Lundborg & Hansson 1980).

Remyelination was found to start as early as the seventh day after crush injury (Nathaniel & Pease 1963) and also after direct nerve suture (Hudson, Morris, & Weddell 1970). When the nerve first starts to regenerate, myelin is seen to be continuous over the surface of the nerve with segmentation occurring later (Vizoso & Young 1948). However, remyelination may not have reached normal levels even at 1 year after injury (Barton 1962).

It was thought that a nerve has a decreased ability to synthesise Schwann cells and regenerate the more proximal the transection injury to it. However experimental studies showed that after transection of spinal nerve roots only a minority of the corresponding neurons did not survive (Meier & Sollman 1978).

In humans the average rate of nerve regeneration is 1-2 mm a day according to various authors, but fluctuates between 1-5mm (Sunderland 1978). Faster rates of regeneration were found in more proximal nerve transections and became increasingly slower with increased distance from the cell body of the neuron.

If these growing sprouts eventually make connections with distal target organs, nerve fibre maturation follows. Even with the best technical ability during repair there will be a reduction in the number of functioning connections compared with normal. Regenerated axons never reach pre-injury diameters and therefore never conduct an electrical impulse as fast. The number of axons that regenerate is seen as a measure of

the success of this process but in the early stages in the distal stump, paradoxically, there may appear to be more. This is thought to be due to the fact that multiple collaterals sprout from each transected axon, they usually grow together in a 'band of Büngner': this is called 'hyperneurotization'. Eventually, the sprouts that reach the end-organ grow in calibre faster than the others in the Büngner band and these regress and. While after successful regeneration some of the axons may reach near normal thickness, the myelin sheaths remain very thin in relation to the diameter of the axon, measured by the G-ratio (Nathan 1987). The extent of myelination also has an important effect on conduction velocity and therefore the function of the nerve fibre and this explains why the conduction velocity in regenerated nerves does not regain previous uninjured values.

It has been shown that regenerating axons grow preferentially toward a distal nerve segment or a nerve graft rather than toward other types of tissue, thereby exhibiting the concept of *neurotropism* (chemotropism) (Cajal 1928). However later experiments by Weiss and Taylor in 1944 provided evidence against this concept but further work since then has produced results contradicting Weiss and Taylor thereby reinstating the existence of 'tissue specificity' (Lundborg et al. 1986). The other thought was that *neurotropism* was due to a 'pruning effect' upon randomly growing axons to the end organ. Neurochemical interactions in the microenvironment form the base for survival of nerve cells normally, as well as in regeneration of the neurons after injury.

Survival of nerve cell bodies after nerve transection is essential for regeneration and is helped by a large number of *neurotrophic* factors from many sources. Lundborg considered the difference between neurotrophic and neurotropic factors (Lundborg

1988). A neurotrophic substance, where 'trophic' means nutrition in Greek, is a substance that influences survival and growth of nerve cells. A neurotropic factor, where 'tropic' means 'way' or direction, is a substance exerting an attraction at a distance, on growing axons. This attraction is likely to be specific to different tissues and in different parts of the body.

Neurotrophic factors can be defined according to their receptors and are usually classified into three groups:

1. The neurotrophins (NGF, BDNF, NT-3, NT-4/5, NT-6)
2. The neuropoietic cytokines
3. The fibroblast growth factors

There are also other groups such as insulin-like growth factor, epidermal growth factor, leukaemic-inhibiting factor, and glial-derived neurotrophic factor.

Methods of artificially enhancing the microenvironment around regenerating nerves may have a role in aiding the process of nerve regeneration. One such way is to infiltrate the nerve repair site with growth factors such as those described above.

Insulin-like growth factor-1 has been shown to increase the rate of axon regeneration in crush-injured and freeze-injured sciatic nerves of rats (Contreras et al. 1995) but a study on rats has shown that infiltrated IGF-1 to the repair site of transected nerves did not improve the recovery of motor function compared to controls nor did it improve accuracy of axonal re-innervation (Lutz et al. 1999). However, brain-derived neurotropic factor and cilary neurotropic factor (Lewin et al. 1997) and leukaemic inhibitory factor (Leong et al. 1999) have been shown to reduce denervation atrophy of target muscles when delivered to the site of epineurial repair of transected sciatic nerves of rats. This was thought to be because stimulation of growth of muscle

and nerve cells was more effective. External application of macrophages may increase axonal outgrowth, probably owing to the factors that they release (Miyauchi et al. 2002; Stolz, Erulkar, & Kuffler 1991).

In 1956 Cohen and Levi-Montalcini isolated nerve growth factor (NGF), a protein that was found to have a neurotrophic effect on the survival of sensory neurons as well as on the outgrowth of their neurites after injury (Derby et al. 1993; Levi-Montalcini 1965). It is usually present in low concentration in the nerve trunk but after injury, levels rise (Saika, Senba, & Noguchi 1991). It is believed that NGF production is triggered by interlukin-1-β released from invading macrophages. It has been shown experimentally that NGF can reduce death of dorsal root ganglion cells when it is applied locally to the repair site of a transected nerve (Rich et al. 1987; Wiberg et al. 1999). However it has no real effect on motor neurons and their neurite outgrowth. Several other researchers furthered the work on the factor, by tracing its effects using a labelling agent. There seem to be many different nerve growth factors that each influence the regeneration of different types of nerve, e.g. it is likely that neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are motor neuron growth factors supporting survival of motor neurons (Henderson et al. 1993).

It was postulated after Cajal's work on the specific growth of growing axons towards the distal degenerated stump and no other tissue, that various other types of specificity could exist, e.g. fascicular or nerve trunk specificity (guidance of regenerating axons to their fascicular origin for instance and reinnervation of the distal peroneal stump by peroneal axons after sciatic nerve repair), sensory and motor specificity (separating their axons), body part specificity (axons going to the appropriate finger after median nerve repair) and end-organ specificity. It was shown

that motor axons tended to reinnervate motor targets, a concept that was thought to be due reabsorption of misguided motor fibre collaterals which followed sensory pathways and maintenance of those which followed motor pathways (Brushart & Seiler 1987).

Other supposed ways to enhance regeneration of nerves such as pulsed electromagnetic fields (Kanje et al. 1993), Sinusoidal magnetic fields (Rusovan & Kanje 1999) and hyberbaric oxygen (Haapaniemi et al. 1998) have also been tried on the sciatic nerve of a rat with variable success.

It is thought that electromagnetic fields increase NGF levels in injured nerves (Longo et al. 1999). There have been studies in rats that have used magnetic fields to aid nerve regeneration. These have had variable results in that the broad conclusion is that axonal regeneration is supported but the mechanism remains unclear (Macias et al. 2000; Sisken, Jacob, & Walker 1995). Polarizing polyethylene tubes with electric charges is also thought to enhance axonal regeneration compared to electrically neutral tubes (Valentini et al. 1989).

Further work is in progress in this field using glass fabric that can be easily wrapped around the repair instead of a tube.

OPERATIVE NERVE REPAIR

The decision to operate on an injured nerve and the technical aspects of any operations have long been a source of considerable debate and is therefore, never an easy one. There have, however, been improvements in the results of peripheral nerve repair since the Second World War which can probably be attributed to the large

amount of research that occurred during this time and the development of microsurgical instruments and sutures, antibiotics and a clearer continued understanding of the structure of the peripheral nerve. Probably the most important step forward in improving the results of nerve repair was the introduction of the operating microscope which made it possible to identify and manipulate nerve structures with better accuracy (Kurze 1964).

Initially the treatment of a nerve lesion depends on accurate diagnosis both in terms of the nerve injured and the site, nature and severity of the lesion. It must then be decided whether to resort to surgical treatment or to proceed with conservative measures. It may be decided that the nerve will not be operated on at all and tendon transfers are the procedures of choice. Operative nerve repair should not, however, replace the necessity for persevering with conservative management until regeneration has stopped and no further improvement is to be expected.

There is no doubt that when the nerve trunk is transected or is only held together by the epineurium (Sunderland Type IV injury), surgical intervention is essential in order to achieve repair before regeneration can take place. This usually involves nerve suture end-to-end if there is not too much contusion that requires excision. The purpose of suture is to align the corresponding fascicular components of the proximal and distal segments to help growing axons achieve the goal of reaching the original endoneurial sheath that contained it. However Edshage showed that perfect alignment of nerve sheaths during suture did not guarantee that growing axons would grow down their original endoneurial tubes (Edshage 1964).

The timing of surgical repair of a nerve has been reported to be important. After nerve severance it was thought that the nerve cell body presents an optimal

metabolic potential 2 to 3 weeks after injury. Results from studies during the Second World War suggested that a 3-week delay was the optimal time for regenerating potential. This allowed time for the debris of degenerated axons and myelin to be cleared away by macrophages, for the synthesis of amino-acids to reconstruct the cytoskeletal structures and Schwann cells to proliferate and guide regenerating axons. It was also thought that delay allowed for better recognition of damaged nerve for neurolysis, and as the epineurium would be thicker it would be better able to hold sutures (Seddon 1954).

Recent work supports the view that much shorter intervals of as little as 2-3 days, may have a positive effect on axonal outgrowth by shortening the initial delay when the cell body switches its primary function from neuronal transport to synthesis of structural components for axonal regeneration (Danielsen et al. 1994; Urabe et al. 1995). It was also reported that if a nerve injury is caused just 2-3 days prior to an additional one, the regenerating fibre grows faster than if no such pre-injury (conditioning) has occurred (Hartree & Hill 1921; McQuarrie & Grafstein 1977). It is thought that after injury, chemical signals are sent by retrograde transport systems to the nerve cell body to trigger the repair process. These 'triggering signals' include depolarization of the cell membrane, absence of substances usually conveyed by retrograde axonal flow, and or presence of substances originating at the site of the injury (both from the axon itself and the surrounding tissues). The cell body up-regulates the synthesis of mRNA and proteins involved in regeneration and cytoskeletal constituents that make up the framework of the axons. mRNAs that are not involved in repair are down-regulated.

Despite much research, agreement has not been reached about the best time for surgery. Some of the most recent studies have shown convincing evidence that the results of nerve regeneration after delayed repair, by means of grafting techniques, compared to that after immediate repair are inferior (Fullarton et al. 2002; Lawson & Glasby 1995). Clinical factors such as the type of nerve, level and site of the lesion, time elapsed since injury, age and health of the patient as well as biological factors such as the time necessary for the cell to react are thought to be important (Brunelli & Brunelli 1990).

There are a number of reasons why delay in nerve repair is detrimental to the regenerative properties of the nerve. First, experimental work has suggested that better recovery of the nerve occurs if repair is carried out between the 20^{th} to 40^{th} day after injury as this corresponds to the time when maximal Schwann cell proliferation is occurring (Holmes & Young 1942). However, progressive shrinkage of the endoneurial tubes, which occurs with increasing periods of denervation, leaves tubes that are so shrunken and fibrosed that they constitute an obstacle to the advance of axons and a threat to maturation of those which do reach and reinneravate peripheral tissues.

Reduction in diameter of the distal stump reduces its cross sectional area up to a half of that of the proximal stump and with increasing periods of denervation the peripheral tissues undergo irreversible changes that ultimately prevent them from responding satisfactorily to reinnervation.

Primary nerve repair compared to delayed repair can be easier at operation because the different tissues can be easily separated while later on the scar involves

all the tissues around the nerve, making the operation much more difficult. Also, two operations give two scars and on the hand this can worsen the quality of recovery.

However, there is much clinical evidence to support the fact that regenerating axons have a great ability to move towards the periphery and, if maintained in a good nutritional state, peripheral tissues have a equally remarkable capacity to respond to reinnervation after periods of time which can amount to years. This is providing new axons enter endoneurial tubes in the distal stump (Seddon 1954).

Sometimes there are other factors that may dictate the time between injury and repair. These relate to:

1. The general medical condition of the patient.
2. Co-existing injuries to other structures, which take priority of treatment e.g. soft tissues, muscles, bones and joints and vascular structures.
3. Delayed wound healing.
4. Residual infection of the wound.

Modern treatment of wounds, with antibiotic therapy and skin grafting, has greatly reduced the time required for wound healing and made surgical treatment of the nerve possible much earlier than previously.

Indications for operation

It can sometimes be difficult to tell the extent of an injury to a nerve when there is no open wound and it cannot clearly be seen that neurotmesis has occurred. Even when there is an open wound and the nerve looks intact, there could be a Sunderland grade III or IV injury that requires excision and repair. Modern gunshot wounds cause severe soft tissue disruption owing to the 'cavitation' caused by the

pitch and yaw of the bullet when it enters a denser medium (e.g. a limb) from the air. The energy transferred from the bullet to its denser surrounding tissues can cause injuries to nerves ranging from neurapraxia to neurotmesis, as well as fractures of the bony elements and vascular disruption. Knowing this, Seddon stated that patients who presented with complete paralysis require exploration, because there was no means of knowing whether the nerve had been completely severed (Seddon 1975).

There are now generally accepted indications for surgical intervention in the event of suspected nerve injury. These indications are based on previous documented surgical experience and the clinical examination of the patient taking into account the mechanism of injury. They are as follows (Birch, Bonney, & Wynn-Parry 1998):

1. Paralysis after wounding or injection over the course of the main nerve.
2. Paralysis after closed injury, especially high velocity, associated with severe soft tissue, bone, arterial or joint damage.
3. Failure of a nerve lesion to recover after the usual expected time for that type of injury.
4. Increasing deficit of motor functions associated with a nerve lesion under observation.
5. Persistent pain at almost any interval after injury. This could indicate abnormal pressure or tension on the nerve.

Conversely, the contra-indications to nerve exploration and or repair are:

1. Other life threatening injuries in need of more urgent attention.
2. Contamination at the site of nerve injury.
3. Evidence of electromyelographic recovery of nerve function after 4 months.

4. Evidence of tension when the two nerve ends are pulled together. This may mean that an interposed graft is required.

Problems with repair

How best to repair a divided peripheral nerve remains an unsolved problem. It is said that the best way to repair a nerve is by direct end-to-end nerve suture without tension (Gutmann & Sanders 1943). This can be difficult to achieve in practice as nerve injuries are often associated with varying degrees of soft tissue loss and the ends of the nerve often retract owing to elastic properties of the endoneurial connective tissue that surrounds each nerve fibre (to form its outer endoneurial sheath), and more importantly the perineurium, whose cells are interspersed with collagen fibres.

Tension

Peripheral nerve axons do not regenerate when the ends of the divided nerve are separated. Gaps greater than only a few millimetres are not supportive of axonal regeneration (Lundborg, Longo, & Varon 1982), whereas gaps of 1-2mm could be supportive to regeneration owing to accumulation of nutrient and white-cell-rich inflammatory fluid (Lundborg, Dahlin, & Danielsen 1982).

It is well known that during normal limb movement a peripheral nerve will accommodate changes in length and tension without affecting the size of the action potentials associated with these nerves when they are stimulated. These findings demonstrate the nerve's elastic properties (Diao & Vannuyen 2000). However it has been shown in animal experiments that nerve suture under tension interferes with

regeneration. There is proliferation of connective tissue at the suture line, which blocks and constricts regenerating axons. This finding led to the practice of using nerve grafts to bridge defects in nerves (Clark et al. 1992; Millesi & Meissl 1981; Terzis et al. 1975). If the limb is extended before nerve union has consolidated, separation of the nerve ends may occur at the suture line. The regenerating axons are then faced with overcoming this gap before entering the distal stump. The nerve can also rupture at sites other than that of the suture line when stretch is applied owing to tethering of the suture site to surrounding tissues.

Stretching a nerve causes intraneural ischaemia. Circulatory changes were shown to occur in the proximal stump of rabbit tibial nerves at only 8% stretch with complete arrest of the microcirculation occurring at 15% (Lundborg & Rydevik 1973). Miyamoto in 1979 showed irreversible results in dog nerve at 5% stretch. Ischaemia causes a reduction in the number of axons being found in the distal stump and a zone of scar tissue being produced in the suture line.

In models of delayed and immediate repair in the sciatic nerves of rats, it was shown that the nerve blood flow in both the proximal and distal segments of the divided nerve, decreased approximately 50% with substantial recovery of function in thirty minutes after an 8% elongation, whereas 15% elongation produced approximately an 80% reduction in blood flow with minimal recovery of its ability function (Clark, Trumble, & Swiontowski 1992). A similar study using an animal model to look the effects of nerve tension or stretch on its blood flow and its conduction velocity showed that the blood flow decreased by a similar amount depending on whether the nerve was stretched by 9% or 16%, but whereas the nerve conduction velocity was not affected in the nerves that underwent 9% stretch, those

that underwent stretch of 16% experienced a gradual loss of two thirds of their starting value. The lack of correlation between blood flow and peak nerve conduction velocity in these studies suggests that ischaemia is not the only factor responsible for an increased nerve latency (Driscoll, Lawson, & Glasby 2002).

Technical ability of the surgeon

The results of operating on nerves depend very much on how skilled the surgeon is. Results obtained by surgeons who regularly do these procedures are much better than occasional operators on nerves (Birch, Bonney, & Wynn-Parry 1998). In today's UK system of surgical training, surgeons are specializing in one sub-type of surgery within a specific branch, i.e. Hand surgery specialist in Plastic or Orthopaedic training. This has the benefits as just described especially if a surgeon happens to be working in a large hospital where many specialists work together. However, not all hospitals are big enough to take on that number of individually trained staff; so many surgeons are general within their field, especially if they take part in an on call rota.

Fasicular alignment

Even if a peripheral nerve is repaired perfectly and successful regeneration of axons across the suture line to end organ targets has occurred, motor and sensory systems are always impaired as severance of a peripheral nerve leads to muscle reinnervation that is abnormal both in degree and specificity. Surgical technique, however, does influence the specificity with which muscles are reinnervated by their original neurone pool (Brushart, Henry, & Mesulam 1981). In order to give a transected nerve trunk the best chance at successful regeneration, it has been shown

that during nerve repair, either end-to-end or interposition grafting, each individual fascicular stump of the proximal end should ideally be sutured to its original fascicular stump at the distal end (Millesi, Meissl, & Berger 1972). This method of repair is technically difficult but leads to more accurate reinnervation than repair by direct epineurial suture.

Interfascicular repair increases the likelihood of the regenerating axons entering the appropriate endoneurial tubes therefore minimizing the chances of interfascicular disorganization but it does not prevent axon disorganization within the fascicle itself (Brushart, Henry, & Mesulam 1981). The mismatch of regenerating axons and their appropriate endoneurial tubes occurs at the suture line, so that when repair involves a graft there are two sets of suture lines to negotiate for the regenerating axons. The cable graft may have an advantage here over muscle or nerve graft, as it allows corresponding fascicles from the proximal and distal stumps to be aligned and joined by a strand of the cable. This should mean that there would be more accurate reinnervation than after other forms of grafting. However it has been shown that there is no difference between the different methods of grafting (Hems & Glasby 1992; Myles, Glasby, & Gilmour 1992).

Reaction to suture material

A foreign body reaction to suture materials can occur at the suture line causing inflammation and fibrosis and resulting in physical obstruction to growing axons entering the distal stump. Adherence of the suture line to surrounding tissues due to inflammation and formation of fibrotic tissue reduces the normal glide of the nerve during movement of the limb. This can cause pain owing to stretching, or fracture of

the nerve at a site other than that of the sit of repair and can be an extremely difficult problem to manage.

History of nerve repair

According to Sunderland (Sunderland 1978), Galen (AD 130-200) was the first to distinguish the difference between nerves and tendons but the first documentation of nerve repair was of that performed by Ferrara in 1608. It was not until the mid 1800s that surgeons became more interested in nerve repair and the early technique included many that now appear totally inappropriate.

Sunderland also quoted that Letievant, in 1873, first described turning back a flap of nerve created from the distal portion of the nerve in a proximal direction, suturing this to a similar flap that was turned down in a distal direction from the proximal end of the nerve (Letievant 1873). This effectively excluded all proximal axons from the site of repair. His cases were reviewed in 1880, and it was shown that no regeneration had taken place. However, he later described simply inserting the distal stump of the divided nerve between the intact nerve fascicles of the proximal portion of the divided nerve in what was called a nerve implantation technique.

Markoe in 1885 described cutting the ends of the nerve stumps obliquely (Markoe 1885), thereby increasing the surface area for nerve contact and in the same year Rawa described side-to-side suture of the nerve ends so the cut surfaces of the nerve were totally left out of the repair site (Rawa 1885). Also described were several suturing techniques resembling those presently used to repair tendons.

Before these times it was generally accepted that either no surgery or amputation was indicated after major nerve severance.

Probably the most significant contributions to peripheral nerve injury and repair studies took place during The Second World War. The studies were commissioned by the Medical Research Council (Seddon 1954).

Methods of repair

The 'gold standard' or commonest form of repair to a peripheral nerve is direct end-to-end suture without tension in an environment free from infection with a good blood supply and good surgical technique. The nerve ends should be cut back until healthy pouting bundles show in the cut surfaces and the corresponding fascicular components of the proximal and distal nerve segments should be aligned as accurately 'as possible during suture (Edshage 1964; Millesi, Meissl, & Berger 1972). However, this is sometimes not possible owing to too large a gap.

End-to-end suture

With the help of the surgical microscope and microsurgical instruments, surgeons have managed to perfect their technique in atraumatic, good fascicular alignment. It has been realized that nerve repair is not just a mechanical problem and despite the best surgical nerve repair, the success in terms of recovery of function of the limb is still unpredictable. At best the surgeon can align individual groups of fascicles, but the behaviour of the separate axons inside individual fascicles cannot be predicted. It seems this is regulated by biological mechanisms at the molecular level.

The theoretical advantages of perineurial (fascicular) repair over conventional epineurial repair (after nerve transection) have been studied by scientists for more than 85 years. Langley was the first to suggest suturing separate nerve bundles in order to secure a better result of sensory and motor function (Langley & Hashimoto 1917). Sunderland also recommended fascicular suture in studies of peripheral topography (Sunderland 1981). Comparison of results from perineurial and epineurial repairs of the sciatic nerve in cats had suggested that return of function of the limb occurred more quickly in the epineurial repair group than in the perineurial repair group (Bora 1978). Salvi retrospectively evaluated 34 patients, who had either undergone epineurial nerve repair early or late, perineurial repair or perineurial sutured graft repair (Salvi 1973). He showed that overall improved function appeared earlier after perineurial repair, particularly in nerves in which grafts were used as opposed to those repaired with the nerve under tension and using standard epineurial techniques.

However, Cabaud compared the results of epineurial and intrafascicular perineurial repair of the ulnar nerves in 20 cats. He was unable to detect any statistically significant difference between epineurial and fascicular nerve repairs and concluded that the epineurial method of repair may be preferable owing to its being technically less challenging to perform (Cabaud et al. 1976). In the 1980s, other studies showed that there was little difference between the 2 types of repair, (Sunderland 1981; Tupper, Crick, & Matteck 1988). Orgel also proposed the use of a modified group fascicular suture (Orgel 1984). Murray et al in 1994 compared peineurial and epineurial repair of transected sciatic nerves in cats and showed no differences in terms of recovery of function of the limb between the two methods of repair (Murray et al. 1994).

In mixed nerves there may be a specific arrangement of the sensory and motor fascicles, in which case it has been suggested that fascicular or group fascicular repair would have an advantage over epineurial repair (Lundborg 1988). Identification of sensory and motor fascicles is a challenge but can be achieved anatomically, electrophysiologically and histochemically. However this is not really feasible in the operating theatre at the present time for a number of reasons: Anatomically, the operating microscope does not have a sufficiently high resolution to identify the fascicles with any accuracy. Electrophysiological testing is possible distal to the repair in the motor nerve but if sensory nerves are to be tested the patient will need to be awake. Histochemistry is reliable but is usually a long process with staining of samples taking up to several hours.

Gluing techniques

The oldest references relating to gluing techniques for nerve repair date back to the 7th century where Aegineta used a combination of 'agglutination' and sutures to repair nerves. In the 13th century Roger of Palma used egg albumin in a similar fashion. The use of fibrin clots to 'glue' nerve ends together was introduced in the early 1940s and showed that some nerve regeneration did occur (Gutmann & Sanders 1942; Young & Medawar 1940). More recently fibrin glues have been developed which have produced good results when compared with epineurial repair (Becker et al. 1989; Narakas 1988; Bertelli & Mira 1993). The glue can be used alone or with one or two stabilizing sutures. The grafts are apposed to one stump and the fluid is dripped on to the union. When the fluid has clotted, the procedure is repeated at the other end. This method avoids trauma to the nerve ends by handling and insertion of

sutures and is less time consuming. However, current concern with transmissible viral disease may make this technique unpopular.

The use of carbon dioxide lasers to weld the epineurium has also been suggested with the theoretical advantage of producing less inflammation, but the results have been variable (Neblett, Morris, & Thomsen 1986).

Nerve grafts

When direct end-to-end suture cannot be achieved owing to tension on the suture line or when considerable mobilization of the nerve is needed (which may devasularize it) or joints need to be flexed in order to bring the two ends of the nerve together, a nerve graft may be interposed. The process of taking the graft triggers degeneration of its strictly neural elements and so the remaining endoneurial tubes of grafts act as conduits and nothing more and guide the axonal sprouts towards the distal axonal stumps.

Nerve grafting includes procedures in which a bridge of nervous tissue is inserted between the cut ends of a nerve. Grafts can, in theory, be pieces of nerve from the same individual (autografts), from another individual of the same species (allografts), or from an individual from another species (xenografts). Grafts can be used fresh or after treatment such as freezing or fixing, after which they are termed bioprostheses. It was thought that nerves sectioned and allowed to degenerate for 8 to 15 days provided a better medium for axon growth than a fresh nerve (Cajal 1928; Duel 1933). This seems to be because the graft was firmer, making it easier to handle and thereby permitting a more effective apposition and union between the opposing faces of the graft and nerve ends (Collier 1953), and not because the axons grow more

quickly through a pre-degenerated graft (Bunnell & Boyes 1939; Huber 1920; Sanders & Young 1942). There was also much work carried out in the 1940s on the use of stored nerve autografts. Experiments conducted using refrigerated or freeze-dried nerve autografts were unfortunately prone to repeated failure and went out of fashion in the early 1970s (Sunderland 1978).

In 1870, work by Philipeaux and Vulpian showed that a segment of lingual nerve could support axonal growth across a gap in the hypoglossal nerve (Philipeaux & Vulpian 1870). This was followed by work by Albert in 1876, which described the first clinical use of a nerve xenograft. He bridged a 3cm gap in a median nerve but the clinical outcome was not reported (Albert 1885). The first successful nerve graft was that performed by Mayo-Robson, who used an allograft to repair a defect in the median nerve (Mayo-Robson 1896). In the early days of nerve grafting, xenografts and allografts were the most popular. Despite favourable results of nerve grafting in the early 1900s (Glasby 1993), scepticism existed regarding their value in nerve repair until the 1970s, as the success of nerve regeneration was not consistent. Poorer results seemed to be obtained with xenografts and allografts compared with autografts (Cao, Shidao, & Yu 1997; Huber 1920), but there was other experimental work that showed useful regeneration of the nerve could occur (Campbell, Bassett & Böhler 1963; Jacoby et al. 1970). Most of the problems with regeneration of axons through allo- and xenografts can be attributed to the intense inflammatory host reaction after their application, thereby hindering axon growth by fibrous tissue formation at the site of regeneration.

After the introduction of microsurgical technique, however, results of grafting did improve. Nerve allo-transplantation was questioned again by Mackinnon in 1996

in a series of experiments, and was subsequently thought to be an option in a carefully selected patient with an otherwise irreparable nerve injury i.e. one where there is so much soft tissue loss, an autograft is not an option, or there is multiple nerve damage (Mackinnon 1996). It should not be forgotten that there was also a vast improvement in the quality of immunosuppressive therapy during this time interval, contributing to the increased feasibility of nerve transplantation (Mackinnon et al. 2002). However this technique has not been widely accepted.

Today, autografts are routinely used for bridging a nerve gap if enough appropriate donor tissue is available. There are two main types of autograft: cable (composed of several strands of a fine usually cutaneous nerve) and full-thickness (a single nerve strand matching the cross-sectional dimensions of the recipient nerve). Although the implanted graft should ideally be of the same calibre as the recipient nerve in order to optimize reinnervation (Seddon 1947), in humans it is difficult to justify using a piece of nerve the same diameter as that of the injured nerve as a graft, as this would mean sacrificing a large motor or mixed sensory and motor nerve. The resultant loss of function in its territory would almost certainly be unacceptable. It has sometimes been possible after severe injury where a second large nerve was damaged and impossible to save.

The method of choice then is the cable graft. The donor nerve is usually the medial cutaneous nerve of the forearm or the sural nerve as these can be sacrificed without significant deficit (Glasby 1990). These strands are aligned parallel to each other into a thicker rope or 'cable', which is sutured or glued without orientating the fascicles, between the proximal and distal stumps of the injured nerve. Although the

cable graft works well as a nerve graft it is awkward and time consuming to construct (Myles, Glasby, & Gilmour1992).

In the past the ends of the cable grafts were sutured randomly to the free ends of the recipient nerve but it has now known that as far as possible, the grafts should be matched fascicle for fascicle at the proximal and distal ends (Millesi 1980; Millesi 1981a). It is thought that this is the most crucial factor in the success of a nerve graft (Hudson et al. 1972). This was the advent of the interfascicular nerve graft and the technique of its application was developed by Millesi in 1981. He identified the fascicle groups of the distal and proximal stumps, matched the fascicular patterns of them and used individual grafts to unite the fascicular groups. He also resected the fascicles, during preparation for repair, at different levels to produce an interdigitated appearance to the repair so that the sutures would be at different levels. These grafts are applied by perineurial suture (Millesi 1981b).

The number of 'neural channels' provided by grafts is many fewer than in the host nerve, so it is important to insert as many strands as the nerve will accommodate. It has also been suggested that the length of the graft should be 15% longer than the gap to be bridged so that there is no tension and the graft lies relaxed (Birch, Bonney, & Wynn-Parry 1998). The results however, of matched diameter (full-thickness) nerve auto grafts have been shown to be superior in humans to those of cable grafts (Bjorkesten 1948; Sunderland 1978c). Despite advances in the techniques of grafting, the results are always inferior to those of direct nerve suture for the following reasons:

1. Technical difficulty or absence of appropriate surgical skill
2. Delay in axon growth (two sites for regenerating axons to cross instead of one)

3. Foreign body reaction to suture material causes inflammation and fibrosis around the site of repair thereby impeding axon regeneration across the junction.

4. The difference in internal structure of the host and donor nerves means that mis-wiring can occur.

5. Associated surrounding tissue damage (potential ischaemia, dirty environment) increases the risk of infection and subsequent inflammation causing fibrosis around the repair.

The difference in the fascicular pattern of the nerve graft compared with the recipient site is an important factor in the determination of the success of regeneration. The fascicular intercommunications result in the gradual dispersal and intermingling of the nerve fibres as the nerve extends distally. Over short distances only a slight modification of fibre-localization occurs. Fibres representing individual branches pursue a relatively localized course in the nerve for variable distances above the site of branching. The amount of change in the fascicular pattern of the nerve and fibre localization increases as it extends distally and Sunderland said that Delangenière had noted that the success of nerve transplantation diminished with increased length of the graft (Sunderland 1978c).

The opposing nerve surfaces have different fascicular patterns, which means regenerating axons will not necessarily enter their original endoneurial tubes in the distal stump even if they manage first to traverse the empty endoneurial tubes of the graft. Some bundles may correspond since not all fascicles are simultaneously involved in plexus formation. However, it should be remembered that this process of axons entering endoneurial tubes is random.

There have been several other attempts by other researchers to address the problem of nerve repair when there is a gap. Stretching the nerve was first described by Assaky in 1886 and followed up by experimental work by Hoen and Brackett in 1955 (Hoen & Brackett 1955). Stretching a nerve causes ischaemia and Wallerian degeneration to occur if the stretch exceeds 40%, leading to loss of function of a limb (Abe et al. 1996). It is thought, however, if a small amount of tension is applied to a healing nerve, the regeneration process is actually stimulated. Nerve tissue adjusts to tension by increasing its compliance if stretched within its physiological limits. Anatomical changes observed in the nerve tissue 7 weeks after stretching apparently accounts for the nerves extensibility, a biomechanical adjustment that helps preserve nerve continuity during the joint extension phase of extremity rehabilitation (Bora, Richardson, & Black 1980).

The role of tissue expansion as an adjunct to nerve grafting has been tried experimentally with some good results. Slow nerve elongation using laser Doppler blood flowmetry controlled expansion in rat sciatic nerves showed that elongation up to 40% was possible with preservation of clinical function. Demyelination did occur but with subsequent remyelination of whole internodes (van der Wey et al. 1995). In another set of rat experiments the mean conduction velocity following tissue expansion of 30%, was reduced to 60% of control values, but by a hundred days this had returned to normal. Histology revealed demyelination without axonal degeneration, which recovered to near normal after the recovery period (Milner & Wilkins 1992).

Other types of graft

Barnes et al in 1946 (Barnes et al. 1946) and Strange in 1950 (Strange 1950) proposed the vascularized pedicle nerve graft as a way to allow the use of full thickness nerve trunks as grafts (Sunderland 1978c). Strange emphasized the importance of maintaining a blood supply to the grafted nerve and developed the ulnar pedicle graft in 1950, which proved to be of value in cases where the median and the ulnar nerves were destroyed in the forearm by ischaemia or extensive soft tissue loss. Vascularized nerve grafts can be 'pedicled,' where the blood supply to the nerve used for grafting is left intact or 'free', where the main artery to the nerve is transected and anastomosed microsurgically to a donor artery in the vicinity of the grafting area. Studies injecting red latex in the subclavian arteries of cadavers have shown that the arterial pedicles to the ulnar, saphenous and deep peroneal nerves and the superficial branch of the radial nerve have mean diameters of over 0.8mm and are suitable for microsurgical anastomosis. Dominant arterial pedicles were not easily identified in 66% of sural nerves and the superficial peroneal nerve had a small supplying artery (<0.8mm) (el Barrany, Marei, & Vallee 1999).

The non-vascularized nerve graft regains its blood supply from the recipient bed whereas the vascularized graft has the advantage of bringing its blood supply with it. Theoretically, the vascularized graft improves results by increasing the number of Schwann cells surviving the procedure, by decreasing intraneural fibrosis, and by increasing the rate of axonal regeneration. However, experimental studies do not support this theory (Shibata et al. 1988). Pedicled vascularized grafts of the sural nerve based on the short saphenous artery (Gilbert 1987), the medial cutaneous nerve of the forearm and the lateral cutaneous nerve of the thigh have also been used with

results as good as each other (Comtet 1983). Thin nerves such as medial and lateral cutaneous nerves of the forearm and the lateral cutaneous nerve of the thigh can either be used as a single strand or as two or three strands arrangement parallel to each other as cable graft if the gap was not too great (Doi, Kuwata, & Sakai 1987) and have been shown to perform better in terms of area of sensory recovery compared to non-vasularised nerve grafts (Boorman & Sykes 1987). Pedicled vascularized nerve grafts are useful in the repair of brachial plexus lesions and give consistent results. The nerve gap can often be greater than 6cm and associated with a large skin defect in this region (Merle & Dautel 1991). Despite much work there is minimal work that demonstrates that vascularized grafts are superior to autografts. The exception may be where nerves are to be grafted in scarred recipient beds and when the nerve injury is associated with a massive skin defect (Doi, Tamaru, & Sakai 1992). Histological results are in favour of vascularized grafts but non-vascularized fascicular grafts placed in a healthy bed recover sufficient neovascularization within a short period of time (4 to 6 days) (Merle & Dautel 1991).

Taylor and Ham extended the idea of vascularized nerve grafting to the free vascularized nerve graft (Taylor & Ham 1976). This innovative technique was proposed to improve the results of nerve grafting but the indications and value of it are not well defined. In a case of total destruction of the median nerve from Volkmann's ischaemic contracture, the superficial radial nerve based on the radial artery was used as a free vascularized nerve graft. At six weeks post-operation the radial pulse was restored and angiography revealed a viable graft with acceptable return of sensation and motor function (Taylor & Ham 1976). Bonney used free-grafting of the ulnar nerve and its artery (Bonney, Birch, & Jamieson 1984);

Breidenbach, 1988 and Birch, 1988 used the ulnar nerve with anastomosis of the ulnar collateral vessels as a free nerve graft (Birch et al. 1988; Breidenbach 1988). Experiments comparing nerve grafting methods of the rabbit peroneal nerve showed that pedicled vascular nerve grafts performed better than free nerve grafts in terms of increased sensory receptive areas and myelinated fibre diameters distal to the grafts (Hems & Glasby 1992).

The search for other materials to use for grafting has gone on for many years, the body's own supply of nerve tissue that can be usefully sacrificed being quite limited. The types of materials tried can be divided into:

- prostheses (synthetic materials)
- bioprostheses (biological materials)
- non-neural autografts

Weiss in the 1940s carried out much work with limited success into the use of non-neural substances as means of providing grafts for nerves (Weiss 1944). However since advances in surgical technique and a greater understanding of nerve regeneration in the past 30 years, work into the revaluation of the use of non-neural substances has been carried out (Lundborg 1988).

Ide pointed out 15 years ago that basal lamina tubes were suitable sites for growing axons (Ide 1984). Any biological tissue containing a basal lamina may be a candidate for use as a bridge for regenerating axons to grow along (Vracko & Benditt 1972). This principle has been used with frozen and thawed muscle grafts used for bridging gaps in nerves and has been tried and found to work well over short distances as a conduit for regenerating axons. The muscle graft has the advantages of being readily available, easy to construct, easy to insert, can be tailored to fit any size

required and provides tubes which are larger in diameter than even the largest nerve fibres, so there is no concern that regenerating motor fibres may be constricted by the smaller diameter Schwann cell tubes present in the donor cutaneous nerves that form the graft (Glasby 1990).

Most of the work on muscle grafting techniques has been performed by Glasby et al (Lawson & Glasby 1998; Mountain et al. 1993), who showed that the muscle graft produces similar nerve regenerative results to the cable graft despite the fact that it is simply sutured into the gap (Glasby 1991). The muscle graft also compares to more conventional methods of nerve repair i.e. nerve grafting, in terms of recovery of function of the nerve. Peripheral nerves in a variety of different animal models, including human subjects have been used in experimental studies with consistent results between them (Gattuso et al. 1989; Gattuso et al. 1988; Glasby et al. 1986; Glasby, Hems, & Pell 1992; Hems, Clutton, & Glasby 1994; Mountain et al. 1993; Norris et al. 1988). According to further work by Hems and Glasby, the muscle graft produced poor results when repair of a nerve defect of greater than 3cm was attempted (Hems & Glasby 1993). This was thought to be due to the fact that Schwann cells, which are important in nerve regeneration, do not have the ability to migrate this distance (Nadim, Anderson, & Turmaine 1990) and that the basement membrane scaffold of the muscle is destroyed by inflammatory cells and replaced by fat and fibrous tissue before regenerating fibres had a chance to grow through it (Hems & Glasby 1992). The importance of treating the muscle tissue by a freeze-thawing procedure has been emphasized as a result of this work. Regenerating axons grow more easily into the basal lamina cylinders of such grafts, which contains

laminin and fibronectin. Migration of Schwann cells into the grafts would be essential for regeneration of the axons to occur over long distances.

Calder and Green used a 'sandwich' combination of muscle graft interposed with a 2mm segment of the distal part of the nerve (Calder & Green 1996). The hypothesis was that the nerve segment would act as a reservoir of Schwann cells, and it was shown that this conduit could support nerve regeneration as well as a conventional nerve graft. This could offer a solution to the problem of bridging a long gap in an important cutaneous nerve, or even a nerve trunk. Thomas found muscle grafts useful for repairing damaged cutaneous nerves in the treatment of painful neuroma (Thomas, Stirratt, & Birch 1994).

Neurotization

Narakas pioneered this technique for brachial plexus injuries (Narakas & Hentz 1988). Neurotization implies nerve transfer or nerve crossover and is broadly equivalent to reinnervation. A healthy donor nerve is separated from its territory and its proximal stump is then connected directly or by means of grafts, usually to the healthy distal stump of the injured or non-functioning nerve. Nerve sprouts grow from the transferred nerve into the denervated nerve elements and establish contact between them and the neurons that formerly supplied another territory. These neurons will now control a peripheral function that was previously performed by different cerebral centres i.e. these neurons now have new end-organ specificity. Sacrificing the donor nerve must be worthwhile; the function gained has to be of greater value than the function lost. The donor nerve should also contain fibres analogous to the recipient nerve, muscle, or cutaneous territory the surgeon wishes to

reinnervate. It is desirable that the cerebral representation of the donor nerve should also have the plasticity to take on the new functions that reinnervation of the injured nerve will restore.

In brachial plexus injuries, extraplexal nerves such as the spinal accessory nerve, rami of the cervical plexus, the intercostobrachial nerve or the intercostal nerves are transferred onto the distal segments of cords, trunks or individual nerves. Or, intact segments of the brachial plexus can act as donor nerves to trunks or cords the surgeon wishes to reinnervate. The latter method is particularly indicated in root avulsions of the plexus in traction injuries. Narakas and Hentz reported good return of elbow flexion in patients with C5 and C6 extraforaminal root avulsion injuries with neurotization using the long thoracic nerve and the accessory nerve in 1988 (Narakas & Hentz 1988).

The first report of neurotization techniques being used for brachial plexus traction injuries was by Harris and Low in 1903 who implanted the distal stumps of ruptured C5 and C6 roots onto the healthy adjacent roots of C6 and C7 (Harris & Low 1903). Several authors since then have described successful transference of intercostal nerves to brachial plexus roots (Millesi 1984; Nagano, Tsuyama, & Ochiai 1989). More consistent good results have been obtained it seems, when plexoplexal nerve transfers have been undertaken (Gilbert & Tassin 1984; Narakas & Hentz 1988).

Entubulation

Various types of tubular structures both biological in origin and synthetic materials have been suggested as conduits to fill nerve gaps, the first dates back to the 1880s where decalcified bone was used as a tube in experimental animals by Glück

(Glück 1880) and Vanlair (Vanlair 1882). Many other types of natural, bioprosthetic and synthetic materials have been suggested over the years with varying success. Arteries were tried with limited success (Huber 1920; Weiss 1943) and the idea became increasingly less popular.

The concept underlying the use of conduits for nerve regeneration is that the regenerating nerve fibres use the endoneurial scaffold of the conduit for directed guidance towards the distal nerve stump, while neuroma formation and ingrowth of fibrous tissue into the nerve gap is prevented (Meek & Coert 2002). The direction of growth and survival of the regenerating axons is also influenced by a chemical effect (the gradient of trophic factors) (Lundborg et al. 1986).

A resurgence of interest in the use of tubes to bridge defects in nerves was brought about by Lundborg when he used mesothelial tubes to bridge experimental nerve defects (Lundborg & Hansson 1980).

Hypothetical advantages to a tube model for peripheral nerve regeneration are:

1. Minimal surgical trauma to nerve ends.
2. Maintaining a gap of 1-2mm between the nerve ends inside the tube is thought to allow for the collection of naturally occurring growth factors. This may improve regeneration.
3. Isolation of the site of repair may allow for chemical enhancement of the regenerative process by manual infiltration with neurotrophic factors.

Isolation of the site of repair may stop axonal sprouts being lost to surrounding tissues instead of growth towards target organs.

4. The tube may prevent fibroblasts from entering the site of nerve repair and so interfering with regeneration.

The mechanisms for the establishment of nerve-to-nerve continuity within a tube after neurotmesis, have been studied by Schroder et al (1993), using a silicone rubber tube model (Schroder, May, & Weis 1993). At three days after the creation of a 1mm nerve gap in a rat sciatic nerve inside the tube, a meshwork of fibrin is formed in the tube bridging the proximal and distal nerve stumps. In this meshwork were erythrocytes, granulocytes, thrombocytes and some macrophages. Fibrin was arranged mainly longitudinally and there was a cleft between the cord of tissue and the wall of the tube, which increased by 7 days after surgery owing to retraction of the fibrin clot. Perineural cells closed the gap between the nerve stumps at 18 days and Schwann cells, fibroblasts and blood vessels occupied the centre of the cord. By 3 weeks after creation of the gap, axons from the proximal nerve stump had reached the distal stump although they were not yet myelinated. It is thought that the perineurium serves as a diffusion barrier from the outside as well as from the inside of the nerve fascicle, therefore providing a defined endoneurial environment for the developing nerve. The similarity between the events, which take place after repair by entubulation, and repair by suture, has suggested a clinical use for the former.

The advantages over end-to-end suture are:

1. Microsurgical facilities and training are not needed.
2. There is minimal trauma to nerve ends.
3. The tube materials are likely to be cheaper than microsurgical sutures.
4. The patient may not need to be transferred to a tertiary referral centre for microsurgical expertise.

Biological conduits

Various authors, in several ways, have tried vein grafts for entubulation. These veins have been used to bridge nerve gaps in rats with some success in regeneration of the nerve (Chiu et al. 1982; Goddard et al. 1984). Walton used vein grafts to repair gaps in digital nerves and showed that the results in terms of return of sensation were similar to those expected from nerve grafting as long as the repair was not delayed (Walton, Brown, & Amatory 1989). Invaginated vein grafts (or inside-out vein grafts) were compared to non-Invaginated grafts by Benito-Ruiz et al in 1994, Kelleher et al in 2001 and Rodriguez in 2001 and showed that invaginating the graft did not make a measurable difference in nerve function compared electro physiologically with non-Invaginated grafts (Benito-Ruiz et al. 1994; Kelleher et al. 2001; Rodrigues Ade & Silva 2001). However, Wang thought that invaginated grafts showed slightly superior results in terms of improved conduction velocity compared with non-invaginated grafts (Wang et al. 1995). Vein grafts filled with fresh skeletal muscle or nerve tissue were thought to be an improved idea over veins on their own as it was thought that if they were filled with an appropriate medium they would have less of a tendency to collapse and be a possible source of basal lamina for axons to grow along. However, the results were similar to those obtained from the vein grafts without a filling substance (Calder & Green 1996; Tang 1995). Some experiments showed regeneration could occur with results being similar to that of conventional nerve grafting (Battison et al. 2000; Brunelli et al. 1993). The use of autogenous vein grafts with intraluminal injection of Schwann cells has been shown to have superior results in terms of regeneration of the nerve compared to vein grafts with added Schwann cells (Zhang et al. 2002). In all the vein graft experiments it was found that nerve regeneration was not well supported if the nerve gap was greater than 2-3cm.

Non-biological conduits

The silicone tube model was developed in 1982 by Lundborg as a tool for studying the physiology of nerve regeneration but subsequent studies showed that *in vivo* persistence of these tubes produces a disturbing inflammatory reaction. However, Lundborg carried on his work with the silicone tube, using it to sheath injuries in the ulnar and median nerves of rats (Lundborg, Dahlin, & Danielsen 1982). In 1997 he performed a study comparing silicone entubulation and suture of the median nerve in a group of 12 patients. In the tube group the nerve stumps were sutured inside the tube with 1 suture leaving a gap of 3-4mm. No significant differences in touch, pressure, vibration, pain, and strength of the interossei muscles between the 2 groups were noted (Lundborg, Rosen, & Dahlin 1997).

Most researchers in this field now agree that a biodegradable conduit is the method of choice if entubulation for nerve repair is to be considered. Non-degradable conduits can cause compression of the nerve and chronic foreign body reactions with excessive scar-tissue formation. These can lead to their need to be removed (Dellon 1994).

Other biological materials such as collagen conduits, some being coated with fibronectin and laminin have been found to support nerve regeneration, but problems with bridging long gaps, invasive scarring, and early resorption have remained (Archibald et al. 1995; Archibald, Shefner, & Krarup 1991).

An early report on the use of a synthetic bioabsorbable polyglactin conduit as a mesh, which was wrapped round a 1cm gap in rabbit tibial nerve, was compared with conventional nerve grafting. The polyglactin produced minimal fibrotic reaction and

was shown to support nerve regeneration (Molander et al. 1982). Polyglycolic acid tubes used to bridge gaps in digital nerves showed good or excellent return of sensation in 86% of patients (Mackinnon & Dellon 1990). In a multicentre study comparing biodegradable tube repair of sensory nerves with standard end-to-end suture, 91% of patients in the tube repair group reported excellent return of sensation compared to only 49% in the end-to-end group (Weber, Breidenbach, & Brown 2000). Teased tendon tissue used as a autograft has been shown to support nerve regeneration judged by return of good muscular tetanic force in rats (Brandt et al. 2002) and alginate gels covered by polyglycolic acid mesh have been used successfully to bridge 50mm gaps in cat sciatic nerves (Suzuki et al. 1999). Several studies have been carried out by Madison looking at the effects of filling a bioabsorbable conduit with laminin gel. He concluded from these that axonal regeneration was supported and its rate was initially increased but was not proportional to the number of axons that became myelinated (Madison et al. 1987). However other experiments at the same time showed that semipermeable tubes filled with collagen or laminin gels may actually impair nerve regeneration through them as significant amounts of the gels were still left in the tubes after 12 weeks (Valentini et al. 1987).

Using a porous collagen tube to bridge a 1cm defect in a nerve has been shown to give better recovery of function than either semi-permeable or non-permeable collagen tubes. This is thought to be because the macropore tube allows neurotrophic factors to gain access to the site of nerve repair (Kim et al. 1993).

Biodegradable glasses are inorganic polymers, normally made of phosphates of sodium and calcium, which completely dissolve in water at a rate that can be selected by adjusting the composition. They provoke little inflammatory reaction and can be

manufactured easily in different sizes to fit different sized nerves. Studies by Gilchrist 1998 showed that these tubes could support nerve regeneration (Gilchrist et al. 1998).

End-to-side neurorraphy

End-to-side coaptation of peripheral nerves has been suggested to have clinical potential in situations in which the proximal segment of a severed nerve is not available. The distal segment of an injured nerve is sutured in an end-to-side fashion to the lateral side of an adjacent nerve trunk. The mechanism of reinnervation after end-to-side neurorraphy is thought to be a result of collateral sprouting at the site of coaptation but remains unclear owing to lack of convincing evidence. True collateral sprouts are those, which grow from intact nodes of Ranvier on the axon with the distal part of that axon functioning normally. Terminal sprouts are those, which regenerate from an injured axon with the distal part of that axon undergoing degeneration. There is no single and clear means of distinguishing these two types of sprout in vivo. Several animal studies have shown that collateral sprouts can occur from intact sensory nerves in response to adjacent peripheral nerve denervation. Most of these studies were carried out by sectioning the sciatic nerve in the rat and observing return of sensation in the denervated skin. This was thought to be due to collateral sprouting of 'C' fibres from the saphenous nerve demonstrated by a dye-labelled plasma extravasation technique. In the intact rat the area demarcated by the dye corresponded to the area supplied by the whole nerve as determined by dissection and electrophysiology. The dye response was used to look at the area of 'C' fibre reinnervation in rats with nerve injuries (Brenan 1986; Brenan, Jones, & Owain 1988). The area of reinnervation was seen to be more widespread if the saphenous nerve itself

had undergone a conditioning or crush injury (Kinnman & Wiesenfeld-Hallin 1993; Wiesenfeld-Hallin, Kinnman, & Aldskogius 1988; Wiesenfeld-Hallin, Kinnman, & Aldskogius 1989). There is also evidence that this collateral sprouting can occur in man. This was seen in the return of sensation in children (Leonard 1973) and adults with unrepaired sensory nerve lesions (Inbal et al. 1987).

It was therefore hoped that this evidence could be extrapolated to motor nerves, and it has subsequently been shown in animal studies over the last 10 years that in end-to-side repair of peripheral nerves (Pieptu et al. 1999; Tham & Morrison 1998; Yuksel, Karacaoglu, & Guler 1999; Zhang & Fischer 2002; Zhang et al. 2000; Zhang et al. 2001), the attached nerve does recover the ability to conduct electrical impulses, the target muscles innervated by the attached nerve recovering up to 60% of their original force of contraction (Lundborg et al. 1994a) and histologically axons have been demonstrated to regenerate at the site of end-to-side coaptation and grow down the attached segment of nerve towards the target muscle. (The details of these studies will be discussed further below.) Clinically, the end point of this repair is to restore as much function to the muscle groups supplied by the injured nerve as possible.

End-to-side nerve repair is not a new idea and dates back over a hundred years ago when Ballance reported using the technique to restore facial muscle function after facial nerve palsy by sectioning the non-functional nerve and attaching it end-to-side to the spinal accessory nerve (1895) or the hypoglossal nerve (Ballance & Ballance 1903). Experimental studies repeating Ballance's work in 1895 suturing the distal end of the facial nerve to the spinal accessory nerve in dogs obtained a return of function of the facial nerve in 3 out of 5 cases (Pieptu et al. 1999). A clinical study was also performed where again, the distal stump of the injured facial nerve was joined to the

ipsilateral spinal accessory nerve using an end-to-side technique, with a reported fair recovery of function. However, there was simultaneous movement of the facial muscles with the trapezius muscle and it was subsequently recommended to use the hypoglossal nerve as the donor for end-to-side repair with the facial nerve (Kennedy 1901). Harris performed end-to-side neurorraphy in cases of brachial plexus paralysis with unsatisfactory results (Harris & Low 1903). These early studies showed that nerve regeneration occurred through an end-to-side repair site in the case of partial injury to the donor nerve. A number of axons are invariably sectioned almost making it an end-to-end repair, or a method of crossover where the donor nerve is not completely sectioned. Poor results led to near abandonment of the technique.

Viterbo, who performed his first set of end-to-side experiments on a rat model revived the concept of end-to-side peripheral nerve coaptation in 1992 (Viterbo et al. 1992). He reported using electrophysiological testing, muscle morphometry and histological examination of sections of the repaired nerve that axonal regeneration could be detected at the distal end of a transected facial nerve after end-to-side neurorraphy. He went on to demonstrate in rats successful motor axonal regeneration using the same tests as above, with end-to-side neurorraphy of the distal end of the peroneal nerve to the side of the tibial nerve through an epineurial window (Viterbo et al. 1994). Lundborg showed in a set of experiments on rats that not only motor but also sensory collateral sprouting could be induced from an intact peripheral nerve (Lundborg, Zhao, Kanje et al. 1994).

The tests that are thought to be useful in assessing the success of end-to-side nerve coaptation are maximum nerve conduction velocity across the coaptation site (to assess whether axons have regenerated past the coaptation site), density of axons in the

nerve trunk distal to the site of repair and myelin and axon diameter (effectiveness of regeneration), and force of contraction of the reinnervated muscle and its mass (assessing whether regenerating axons have made effective connections with their muscle fibres).

Since Viterbo's first experiments in the early 1990s there has been marked interest in the field with much research having been produced into ways of refining the technique in an attempt to try and improve results.

There are a number of issues that have arisen regarding the success of end-to-side neurorraphy since the resurgence of interest in it in the last 5 years, and are as follows:

Predegenerated versus 'fresh' distal nerve stumps

Lundborg compared end-to-side suture of predegenerated segments of sciatic nerve in rats with a fresh end-to-side attachment and found with neurofilament staining and histological examination that sensory axons were present in the attached nerve segment of the 'fresh' group to a lesser extent than the predegenerated group. The 'pinch' test (which involves watching for involuntary skin movement on the rat when an area of skin is 'pinched'), was also performed on both groups to show the extent of sensory reinnervation, and seemed to evoke more positive results in the predegenerated nerve segment group compared to the fresh nerve segment group (Kerns et al. 1993).

True collateral sprouting

Although histological studies of these repairs have shown that axons do sprout from the donor nerve into the distal stump of the attached nerve, it is still

unclear whether this is a result of damage to the donor nerve (i.e. in the suture process and or the making of the epineurial window) or due to true nodal collateral sprouting from intact axons from the donor nerve.

In 1899 Kennedy described end-to-side coaptation where the donor nerve's epineurium was incised and a fascicle deliberately transected. He then coapted the distal stump of the facial nerve to the side of this partially divided nerve (hypoglossal) and reported facial reanimation (Rovak, Cederna, & Kuzon 2001). In this case of end-to-side repair with deliberate axotomy, the distal cut portion of axon undergoes Wallerian degeneration with subsequent impairment of function of this nerve. Regenerating proximal sprouts may enter either the endoneurial tubes of the distal part of the donor nerve, or the endoneurial sheaths of the attached recipient nerve. Motor unit remodelling in the remaining intact donor nerve motor end organs probably compensates for the loss of motor axons that become diverted through the end-to-side graft. This remodelling may decrease or eliminate any donor nerve functional deficit (Cote & Faulkner 1984). This is an example of terminal sprouting.

Ballance and Ballance (Ballance & Ballance 1903) are credited with reporting the first end-to-side coaptation without donor nerve axotomy. They described five clinical cases of restoration of voluntary facial muscle function following suture of the cut distal end of the facial nerve to the intact side of the spinal accessory nerve. There was corresponding movement of the trapezius muscles and the sternocleidomastoid muscles in all cases following the repairs (Rovak, Cederna, & Kuzon 2001).

Lundborg performed end-to-side suture of the transected distal stump of the peroneal nerve of the rat that had been left to degenerate for seven days, to the ipsilateral tibial nerve. He demonstrated restoration of 60% of the contractile force of

the tibialis anterior muscle, which is supplied by the peroneal nerve, showing that foreign motor axons (from the tibial nerve) have been attracted into the distal stump of the attached peroneal nerve and subsequent muscle reinnervation (Lundborg et al. 1994). He wondered whether these regenerated axons represented collateral sprouts or regenerated axon tips of fibres, which were damaged during or after the attachment of the end-to-side segment. In the latter group, degeneration should be seen distal to the site of repair. It is known that 100% contractile force is restored by only 20% of axonal reinnervation. Considering that 60% of the contractile force was restored, this degeneration in the donor nerve should have been massive, but signs of it were minimal even when a perineurial window was introduced. He therefore assumed that many axons in the attached segment were derived from collateral sprouts. The figure of 60% restoration of denervated muscle strength was matched in 1998 (Tham & Morrison 1998). Sananpanich reported 50% of the original number of axons are present in the recipient nerve and the muscle mass is 50% of the contralateral control (Sananpanich, Morrison, & Messina 2002).

Another way of looking for collateral sprouts is to perform retrograde labelling studies of the attached nerve stump to assess axon growth at the end-to-side junction. A study using horseradish peroxidase axonal tracing to label motor neurons histologically in the spinal cord and sensory neurons in the dorsal root ganglia, demonstrated axonal sprouting from intact axons. The technique showed that the reinnervating axons did not sprout from the two spurious sources of axonal regeneration (recipient nerve axons cut at a different level, or donor axons injured at the site of end-to-side repair), but mostly from intact neurons (Chen & Brushart 1998).

Epineurial barrier to regeneration

There have been studies comparing the axonal growth from donor to attached nerve with or without using an epineurial window and some of these seem to show that the epineurium can act as a barrier to axonal sprouting. Some authors in several series of experiments considered this. Some studies have concluded that the epineurium did act as an inhibitor to reinnervation (Bertelli, Soares dos Santon, & Calixto 1996; Noah et al. 1997a & b). In the former study a nerve graft was used to connect the proximal median nerve stump end-to-side to the distal stump. Fibrin glue was used for the repair and the epineurium was not removed. There was no evidence of sensory or motor recovery. The latter study compared end-to-side neurorraphy in rats with the epineurium intact, removed and both epineurium and perineurium removed. The largest numbers of regenerating axons were found in the group with both layers removed suggesting the perineurium acts as a diffusion barrier to NGF and other neurotrophins. Other studies have suggested that that regenerating axons can penetrate the perineurium (Viterbo, Trindade, Hoshino, & Mazzoni 1994)and all three layers (endo-, peri-, and epineurium), but axonal counts are increased if an epineurial window is made at the site of end-to-side coaptation (Viterbo, Trindade, Hoshino, & Mazzoni 1992) (Cao, Shidao, & Yu 1997; Lundborg et al. 1994a; Zhao, Chen, & Chen 1997).

It should be noted that the risk of donor nerve axonal injury is much higher when removing perineurium and epineurium rather than just the epineurium.

The role of neurotrophic substances

The survival of nerve cells depends on a trophic influence from peripheral target cells. These target cells normally supply chemical signals to their innervating

cells. Signals are transported retrogradely along the axon and used to sustain essential functions of the nerve cell body (Varon, Manthorpe, & Williams 1984).

It is known that the source of the signal for terminal sprouting (but probably not for nodal sprouting), is denervated or otherwise inactivated muscle fibres, whose action is boosted by the presence of degenerating nervous tissue (Brown, Holland, & Ironton 1980). Evidence shows that uninjured mature neurons are normally quiescent but maintain their capability for continued growth for many years (Isaacson, Saffran, & Crutcher 1992; Ruit et al. 1990).

There is then the question of how the axons inside the donor nerve are able to penetrate the endoneurium, perineurium and epineurium. After nerve damage, Wallerian degeneration occurs distal to the injured site. Therefore degenerating axons at the cut end of the distal nerve segment undergoing end-to-side suture could produce growth factors, which provide a supportive environment for regenerating axons (Baron-Van Evercooren et al. 1982; He & Chen 1992). Factors such as NGF, CTNF and bFGF have been found to induce collateral sprouting from uninjured axons *in vivo* (Gurney, Yamamoto, & Kwon 1992; Isaacson & Crutcher 1998) but newly sprouted axons do not persist after the cessation of a NGF infusion (Isaacson & Crutcher 1995). Local Infusion of IGF-1 may speed up return of function of the denervated muscle by increasing the rate of axon regeneration, according to a study of end-to-side nerve coaptation in rats (Tiangco et al. 2001). McCallister infiltrated the epineurial sheath 1cm proximal to the end-to-side coaptation site with NGF and CTNF and showed that axon density was increased compared with that seen in end-to-side coaptation without nerve growth factor injection (McCallister et al. 2001a).

Despite these results the role of artificially added growth factors to repaired nerves still remains controversial.

Proximal stump contamination

It has been shown in the numerous studies discussed above that the end-to-side attached nerve segment can attract sensory and motor axons from an intact nerve. McCallister wondered whether these mostly good results could be due to contamination from the proximal stump of the severed nerve undergoing end-to-side neurorraphy (McCallister et al. 2001). In the histological examination of their end-to-side nerve repairs in rats they noticed in a cross-section of the nerve taken proximal to the coaptation site and distal to the peroneal stump regenerating axons travelling in the outer epineurium of the tibial nerve proximal to the coaptation site before re-entering the grafted peroneal nerve. This could represent regenerating axons originating from the proximal stump. This is not to say, however, that axonal sprouting does not occur at the site of end-to-side coaptation at all as there is a wealth of other studies that demonstrate good return of nerve conduction and return of muscle function when care was taken to ligate the proximal nerve stump with a ligature and bury it into nearby muscle (Giovanoli et al. 2000; Liu et al. 1999; Tham & Morrison 1998; Zhang et al. 1998; Zhao, Chen, & Chen 1997).

Effect on the donor nerve and muscle

When the recipient nerve is sutured to the side of a donor nerve in an end-to-side neurorraphy it can be hypothesized that physical disruption of axons occurs at the site of nerve coaptation, even though there is no intentional axotomy. However if axotomy occurs of motor fascicles there will be acute denervation of some of the

muscle fibres in the donor muscles. If this muscle denervation were significant, a force deficit would be expected and this would therefore limit the feasibility of this technique in clinical practice.

Most of the recent studies on end-to-side nerve repair report negligible effects on the donor nerve. Even when a perineurial window was made in the donor nerve of a rat, potentially traumatizing the donor nerve fascicles, there does not seem to be any degeneration distal to the site of coaptation which causes any significant muscle atrophy or loss of muscle function (Liu et al. 1999; Viterbo et al. 1994b). However, a recent study reported a loss of 12% of the force of contraction of the muscles innervated by the donor nerve compared to normal. Sananpanich showed that although there was no change in the number or mean size of myelinated axons, there was a decrease in the mean myelin thickness (Sananpanich, Morrison, & Messina 2002). Cederna peformed end-to-side experiments in rats and looked at the acute and chronic effects of the repair on the donor muscles by assessing their mechanical properties after 2 and 6 months. They found that acutely, the end-to-side neurorraphy caused denervation of the donor muscles suggesting axon disruption. However in the longer term the structure and function of the donor muscles returned to normal (Cederna et al. 2001).

Different spinal cord levels

According to a study by Zhang, end-to-side nerve repair cannot be used to repair nerves when the donor and recipient nerves originate from different spinal cord levels. He attached the distal stump of the obturator nerve (L2-4) in an end-to-side fashion to the sciatic nerve (L4-6) and reported no visible axons at the coaptation site. When the peroneal nerve was attached to the posterior tibial nerve (both derived from

L4-6) viable axons were identified in the peroneal nerve distal to the site of neurorraphy (Zhang et al. 1998). Sananpanich attached the distal stump of the musculocutaneous nerve to the ulnar nerve with significant recovery of function of the biceps muscle (Sananpanich, Morrison, & Messina 2002). These two nerves are derived from mutually exclusive parts of the spinal cord. This is an encouraging result, as in many of the previous studies that reported a good capacity of collateral sprouts from intact axons to innervate target organs in end-to-side neurorraphy, the distal end of the cut nerve was sutured to its parent nerve up to only 1.5cm distal to the original branch (Liu et al. 1999; Noah et al. 1997; Tham & Morrison 1998; Viterbo et al. 1994; Zhang et al. 1998; Zhao, Chen, & Chen 1997). In cannot be excluded in these circumstances that reinnervation occurs retrogradely from original axons, especially as the injury may excite regenerative responses. It has been shown that nerve injury and degeneration is a signal for axonal sprouting (Brown, Holland, & Ironton 1980).

SPECIFIC AIMS OF THE PRESENT STUDY

At present, the results of end-to-side repair of an injured nerve have shown it to be useful only as a salvage procedure in restoring muscle function. The vast majority of this end-to-side experimental work has been carried out only on rats (peroneal to tibial nerve). These have shown that end-to-side repair can support nerve regeneration with restoration of good function to a limb comparable to that, which would be expected with a conventional nerve graft. Clinical studies have been carried out on patients with brachial plexus lesions who have undergone end-to-side implantation of the injured nerve root into an adjacent root, but again the results of these have not been consistent (Kostakoglu 1999). A recent study by Ferraresi used

the hypoglossal nerve as a donor nerve for end to side coaptation with stumps of various parts of brachial plexus in patients with multiple avulsions of the plexus. They did not report any useful function of the reinnervated muscles although they did get electrical conduction down the regenerated nerve (Ferraresi et al. 2002).

There have been no experimental studies on larger animals whose nerves, in size approach those of humans. Large primates are an obvious choice especially as the function of the hand and distribution of sensation in it is similar to that of humans. In reality the availability, cost and keep of these animals is such as not to make the study cost-efficient. Sheep are a good experimental model to use in place of primates. They are relatively cheap to buy, are in good supply, are easy to handle and keep, and their nerves are of similar calibre to those of humans.

End-to-side nerve repair has emerged, as a potential solution to the drawbacks of neurotization and tendon transfer techniques as it does not involve the sacrifice of other normal nerves or tendons. In theory, it allows the growth of new neurons by way of collateral sprouting from the donor source, into the attached distal stump of the injured nerve. They continue regenerating distally; sufficiently to re-establish end organ contact to reinnervate a muscle or group of muscles that would normally be supplied by that injured nerve. Despite a large amount of research in this area, there is still no concrete evidence that true collateral sprouting as opposed to terminal sprouting is occurring from the donor nerve to the attached distal stump of the recipient nerve.

Sheep do not react well to HRP (horseradish peroxidase), a substance that has been used in previous small animal studies for retrograde labelling techniques. These techniques can be useful in such studies to trace the origin of the regenerated

axons in the distal attached nerve stump to the end-to-side coaptation site. It is therefore not ethical to use HRP on these animals so an alternative way to look for collateral sprouting is to assess the donor nerve electrophysiologically and histologically. The FCR muscles supplied by the attached distal stump of the median nerve were assessed physiologically to look at changes in peak forces of contraction compared to normal muscles. The distal stumps of the attached median nerves were assessed morphometrically to look for evidence of damage and subsequent healing of the nerves, e.g. smaller nerve fibre diameters, slower conduction velocity and decreased muscle mass. Theoretically, if the donor nerve was not damaged during end-to-side attachment and conduction from the donor nerve to the distal nerve stump is seen to occur, this could suggest that this conduction is due to collateral sprouting. Terminal sprouting is what one may expect to find if the donor nerve had been damaged during recipient nerve stump attachment and regenerating axons may enter either the endoneurial sheaths of the distal donor nerve, or the endoneurial sheaths of the recipient nerve.

F wave studies involve the antidromic electrical impulse that travels proximally along the nerve in the direction of the spinal cord from a distal stimulus. These impulses are reflected back from the spinal along the nerve again in an orthodromic direction to produce a second smaller muscle contraction, which manifests as a smaller CMAP (compound muscle action potential) on the osillocsope screen (F wave).

If the distal stump of the attached median nerve is stimulated distal to the neurorraphy site, the impulse will travel to the to the neurorraphy site and then in both directions to the spinal cord and to the FCU muscle via the donor ulnar if

collateral sprouting had occured. An F wave will be recorded when the impulse returns from the spinal cord back along the donor ulnar nerve to contract the FCU muscle. Impulses through terminal sprouts will theoretically not travel in a distal direction to the donor FCU muscle at the neurorraphy site to produce a muscle contraction before the F wave stimulated contraction, only antidromically to the spinal cord, followed by the F wave. Impulses arriving at a junction where there is collateral sprouting could produce more than one F wave response due to the stimulus travelling ortho and antidromically.

It has been shown successfully in studies on rats that injuring a nerve proximal to a site of nerve regeneration can enhance axon growth (Bisby & Pollock 1983; Jacob & McQuarrie 1993; Sjoberg & Kanje 1990). The model that incorporates this idea presented in these experiments is the double end-to-side experiment. It is possible that nerve axons may use the 'bridge' section of this model as a conduit in order to grow towards the distal attached median nerve stump and reinnervate the end-organ (FCR muscle) (McCallister et al. 2001b)

The aims of this study are to compare the capacity for and extent of nerve regeneration for a variety of different methods of nerve repair that are either clinically accepted or recognised to support nerve regeneration experimentally on a sheep model. These will be compared in terms of electrophysiological tests, morphometric muscle testing and histological examination of the regenerated portion of the nerve.

Summary

- To look at the efficacy of end-to-side nerve repair in a large animal model.
- To compare end-to-side neurorraphy to conventional methods of nerve repair.
- To elucidate the mechanism by which reinnervation of an end-organ from an end-to-side neurorraphy takes place.
- To look at the effects of coapting the proximal nerve stump end-to-side to the donor nerve a distance proximal to the distal end-to-side neurorraphy site.
- To assess the extent of donor nerve damage.

CHAPTER 2 — MATERIALS AND METHODS

ANIMALS

The sheep was chosen as the experimental animal for the experiments in this thesis. Most of the work previously carried out on end-to-side nerve repair has involved rats or rabbits with some good results. Although rabbits were used extensively for peripheral nerve research by Sanders and Young during the Second World War (Lawson & Glasby 1995), their peripheral nerves are of small calibre and do not correspond well with those in humans. The same is obviously true for rats. When rapid answers to questions not involving time are needed, these animals are an ideal experimental tool, but when time plays a part in the experiment, larger animals with properties similar to those of humans are better. Rabbits and rats can be expensive and this may result in restricted group size.

It was therefore felt that there was need for an end-to-side model where the nerve trunks and time-course for recovery related more to humans. The general properties of nerves of the sheep are similar to those of the human in terms of calibre and length therefore making the regeneration distances similar (Drew et al. 1995; Fullarton et al. 2001; Fullarton, Glasby, & Lawson 1998; Glasby, Fullarton, & Lawson 1997).

Home Office regulations state that a minimum number of animals that will allow statistically significant results in experimental work carried out on them should be used (Home Office (H.M.S.O.) 1986). Power studies were performed to estimate a suitable group size based on previous results (Fullarton, Myles, Lenihan, Hems, &

Glasby 2001). The details of these are outlined in the 'statistics' section of this chapter.

EXPERIMENTAL GROUPS

The object of the present experiment was to compare normal sheep's median nerves, electrophysiologically and histologically with sheep's median nerves that had undergone neurotmesis and repair by means of specific surgical techniques. Also the morphometry and weights of the flexor carpi radialis muscles (supplied by the median nerve) were compared for normal and experimental sheep.

The experimental groups were the end-to-side and double-end-to-side groups of sheep, which were the procedures undergoing experimental evaluation in this work. In order to make a fair assessment of these procedures for possible future clinical use in restoring function to a limb, it was thought that they should be compared to other acceptable ways of nerve reconstruction as well as to normal nerves and muscles. These conventionally applied clinical techniques were the *'conventional repair'* groups of sheep. In addition a comparison was made with entirely normal animals — the '*normal*' groups.

It was also appropriate to compare normal ulnar nerve function and flexor carpi ulnaris (FCU) muscle function in a similar manner to the median nerves and flexor carpi radialis (FCR) muscles. This was to test for damage to the ulnar nerve and subsequent compromise of function of FCU during end-to-side neurorraphy in these experimental groups. The ulnar nerve supplies the FCU muscle in sheep.

Normal groups

There were two *normal control groups* each consisting of 8 sheep that underwent no operation. These were termed Group1 '*median controls*' and Group 2 '*ulnar controls*'. The animals were matched for age with those used in the experimental groups at the time of their assessment.

These groups underwent subsequent electrophysiological and histological assessment of the respective nerves and morphometric analysis and wet weight measurement of the muscles these nerves innervated.

The idea of a sham operation was discussed before the experimental study plan was devised. It was not felt that using extra sheep for this type of experiment would concur with Home Office guidelines and that the above normal group experiments would provide adequate normal values with which to compare the results of the other experiments.

Conventional repair groups

Previous work has shown that nerves repaired with clinically accepted techniques allow nerve regeneration to a certain clinically accepted standard. There is minimal differences between them in terms of the maximum motor conduction velocities and the function of the muscles they innervate. One of the aims of this work is compare the end-to-side new experimental technique for nerve repair against accepted conventional methods of nerve repair. For this purpose these groups of sheep were called *conventional repair.* The *conventional repair groups* each consisted 6 sheep, which underwent the following procedures:

- Group 3: **Neurotmesis.**

The median nerve was sharply transected 5cm distal to the ribcage of the sheep, with a neurotomy knife and immediate end-to-end epineurial suture was performed.

- Group 4: **Nerve graft.**

Sharp transection of the median nerve occurred as above in order to remove a 1cm section of the median nerve at the same position along the nerve. The 'graft' was turned 180° and immediately sutured back into the defect created using epineurial sutures at both distal and proximal ends.

- Group 5: **Controlled release glass (CRG) entubulation.**

This biodegradable glass fabric, discussed in the previous chapter, is an example of a nerve conduit that aids nerve regeneration by providing regenerating axons with a pathway to guide their growth towards the distal stump of the nerve. Biological conduits such as vein and collagen as well as synthetic ones such as glass, silicone and polyglycolic acid, have been shown experimentally and clinically to support nerve regeneration. Here a piece of the glass fabric was wrapped round the nerve, to form a tube to enclose its transected ends.

Experimental groups

The two experimental groups each consisted of 12 sheep and were termed *'end-to-side'* and *'double end-to-side'*. The sheep underwent the following operations:

- Group1: **End-to-side.**

Neurotmesis of the median nerve and immediate end-to-side coaptation of the distal median nerve stump with the intact ulnar nerve.

- Group 2: **Double end-to-side.**

Neurotmesis of the median nerve and coaptation of both stumps of the median nerve 1cm apart to the side of the ulnar nerve. This 1cm length of donor ulnar nerve was called the 'bridge'.

A table outlining how the groups of sheep were organized is set out below:

Group	**Surgical procedure**	**Experiment**	**No. of sheep**
1	None	Median nerve Normal	8
2	None	Ulnar nerve Normal	8
3	Median nerve neurotmesis	Conventional repair	6
4	Median nerve graft	Conventional repair	6
5	Median nerve glass wrap	Conventional repair	6
6	End-to-side neurorraphy	Experimental repair	12
7	Double end-to-side neurorraphy	Experimental repair	12

Fig 1 Table to show the groups of sheep involved in the experiments in this thesis.

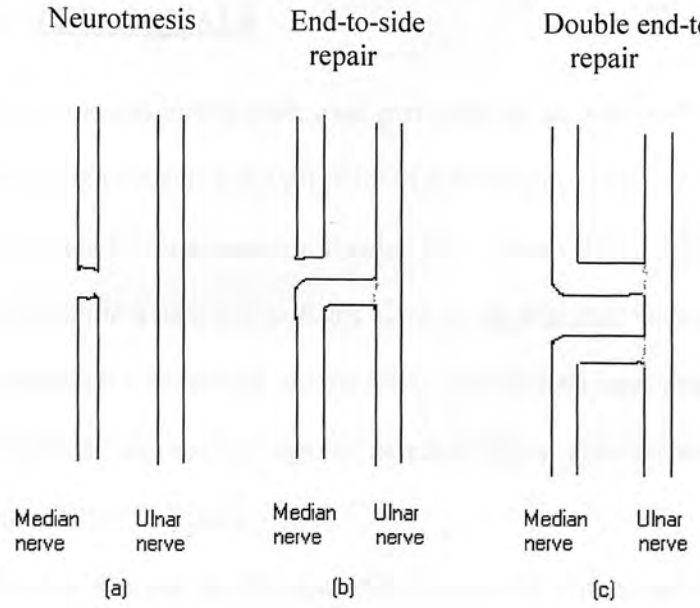


Fig 2 Diagrammatic representation of the end-to-side neurorraphy procedure:
a. Neurotmesis of the median nerve.
b. End-to-side coaptation of the distal stump of the recipient median nerve to an epineurial window made in the side of the donor ulnar nerve (End-to-side model).
c. Double end-to-side model. The proximal stump of the median nerve is coapted to the side of the ulnar nerve as well as the distal stump.

CARE OF THE ANIMALS

All experiments in this work were performed at the Marshall building which is Medical research council and University of Edinburgh owned. This is situated at the Roslin Institute for Bioresearch at Roslin. It is a Home Office approved site. The animals were kept in a barn and in fields close by the Marshall building. They were fed once a day with a pellet type animal feed. The animals were inspected daily in the field to look for any obvious signs of illness or injury and were weighed every 4 weeks (Home Office (H.M.S.O.) 1986).

All sheep selected for the experiments were of a similar age (about 2 years) and of an average weight of 60kg. They were fasted the night before surgery and isolated in a clean pen.

Anaesthesia

For induction of anaesthesia one side of the neck of the sheep was shaved with animal clippers in order to locate the jugular vein. A 1% solution of thiopentone (1ml/5kg) was injected into the jugular vein in a caudal direction. An animal handler held the animal firmly during this procedure until it succumbed to the effects of the induction agent. The animal was lifted onto a trolley by four animal handlers and then intubated. The vocal cords of the animal were directly visualized using an animal laryngoscope and an endo-tracheal tube was passed through them. The cuff of the tube was inflated with 10ml of air and a stethoscope was used to check that the lungs could be inflated through the endotracheal tube. The animal was moved into the operating theatre and placed on its back on the operating table and secured with side restraints. Anaesthesia was maintained using inhaled 1-2% halothane, delivered with a mixture of

6 litres per minute of oxygen and 2 litres per minute of nitrous oxide from a pressure-controlled ventilator (BOC-Manley, Promovent).

The right upper limb was stretched out and secured in this position using a gutter type restraint and adhesive tape (Sleek, Schering).

Perioperative monitoring

A distal patch of hair measuring 3x6cm above the sheep's hoof was shaved for three limbs of the animal for ECG monitoring and the tongue was used for oxygen saturation monitoring. A temperature probe was also inserted into the animal's oesophagus.

Signs of depth of anaesthesia such as the degree of dilatation of the animal's pupil (having previously noted the pupil size in a normal healthy awake animal) and the blink reflex by stroking the animal's eyelashes were monitored. The amount of inhalational anaesthetic, oxygen and nitrous oxide that was being administered by the anaesthetic machine was checked. A stethoscope was used to check the animal's lungs were still being inflated adequately and the cuff of the endotracheal tube was inflated adequately forming a seal so no air could escape. The animal's heart rate was watched on the monitor and checked at the carotid pulse periodically. Percentage oxygen saturation was watched on the monitor readout connected to the oxygen saturation probe and the colour of the sheep's tongue was also monitored as a sign of oxygen desaturation.

SURGERY

Exposure

The surgical exposures of each nerve for each group were the same. The volar surface of the right upper limb was shaved right up to the thoracic cage. On stretching out the limb, the pectoralis muscle became tented which acted as a landmark for correct placing of the surgical incision. The shaved skin over the arm was prepared with antiseptic Betadine solution and square draped so that only a small area 10 x 10 cm was visible. A longitudinal 10cm incision was made in the skin just rostral to the most tented part of the visible pectoralis muscle 10cm from the mid point of the sternum and deepened down through subcutaneous fascia. The pectoralis muscle was divided bluntly in line with the fibres to reveal loose connective tissue, which revealed the neurovascular bundle lying on a flat bed of muscle and bound to it by a layer of tough connective tissue. The bundle consisted of the median nerve, the ulnar nerve lying just next to it and the brachial artery and vein. The median and ulnar nerves were sharply dissected free from the connective tissue. Haemostasis was maintained throughout using bipolar diathermy. Microsurgical division of nerve was achieved using a small 1cm length disposable scalpel blade mounted on a plastic handle.

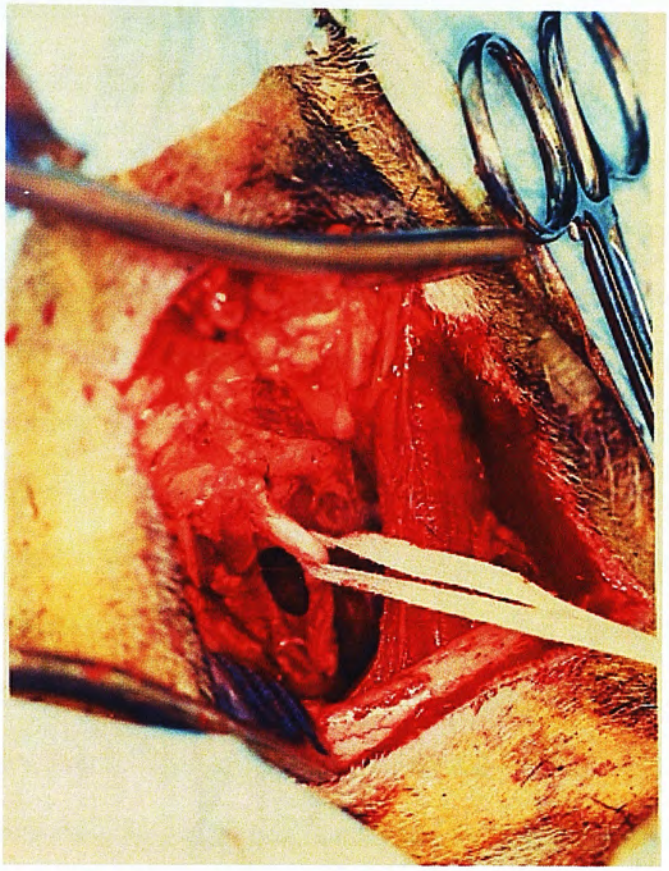


Fig 3 Picture to show the dissection of the median nerve, which is held in the cotton sling. The divided pectoralis muscle can be seen to the right of the nerve.

Group 3: Conventional repair– neurotmesis

The median nerve was divided sharply about 6cm from the rib cage with a small scalpel blade, directly in the line of vision. With the transected nerve lying on a piece of material of contrasting colour the cut ends were aligned as accurately as possible. Any fraying of the nerves ends or protruding ends of fascicles were trimmed using the sharp scalpel blade. Intraneural bleeding was controlled using cotton buds. Repair took place using 6 epineurial interrupted 9-0 polyamide (Nylon) sutures (Ethicon Ltd, Edinburgh,UK) with vision aided by means of an operating microscope (Wild Heerbrugge M600). Microsurgical instruments were used. The epineurium is the outer most covering of the nerve trunk and is easily identified when it is breached as it is thick and distinguishable from the deeper coverings of the nerve and also bleeds from vessels that lie within it. The most posterior suture was placed first and then two anterolaterally to form the points of an equilateral triangle. Three more sutures were then placed at equal distances between the first three, finishing with the most anterior suture. Sutures were placed so that the knot of the suture lay outwith the epineurium The microscope had stepless variable magnification of 3.5 to 18 times and was used for all the operations where epineurial nerve suture took place.

Group 4: Conventional repair - nerve graft

The median nerve was sharply divided as above and a 1 cm section of nerve was completely removed. This section was turned 180° and placed back into the nerve defect. This piece of nerve acts then, like a nerve graft, as the fascicle ends will not match up as well as when the two ends of a simply transected nerve are simply

repaired in alignment. Both ends of the graft were sutured into place using 6 9/0 nylon interrupted epineurial sutures at each end using an operating microscope to aid vision.

Group 5: Conventional repair – CRG wrap

After exposure of the median nerve as for the other experiments above, sharp transection of the median nerve in the same position as for the above procedures, took place. A rectangle of size 5cm by 3cm of CRG fabric was cut and positioned such that 1-2 cm would lie above and below the neurotmesis with a good overlap of 1cm of the edges once the nerve ends had been 'wrapped' in it. This was approximately 4x3 cm.

The fabric was placed under the two cut nerve ends and the ends of the nerve were apposed as far as possible, gently with non-toothed forceps. Glass polymer glue was placed on one the edges of the fabric and the other edge was then folded over the cut nerve ends and stuck down onto the edge containing the glue. The glass polymer glue is made up of the same components than that of the glass fabric. Finger pressure was used to hold the wrap in place for a few seconds to allow the edges of the fabric wrap to adhere together. A blob of fibrin glue (Tisseel, thrombin powder and Calcium chloride solution, Baxter Healthcare Ltd.) was placed at each end of the wrap tube to secure the ends of the nerve in place. Care was made to ensure no polymer or fibrin glue leaked into the nerve transection/regeneration site.

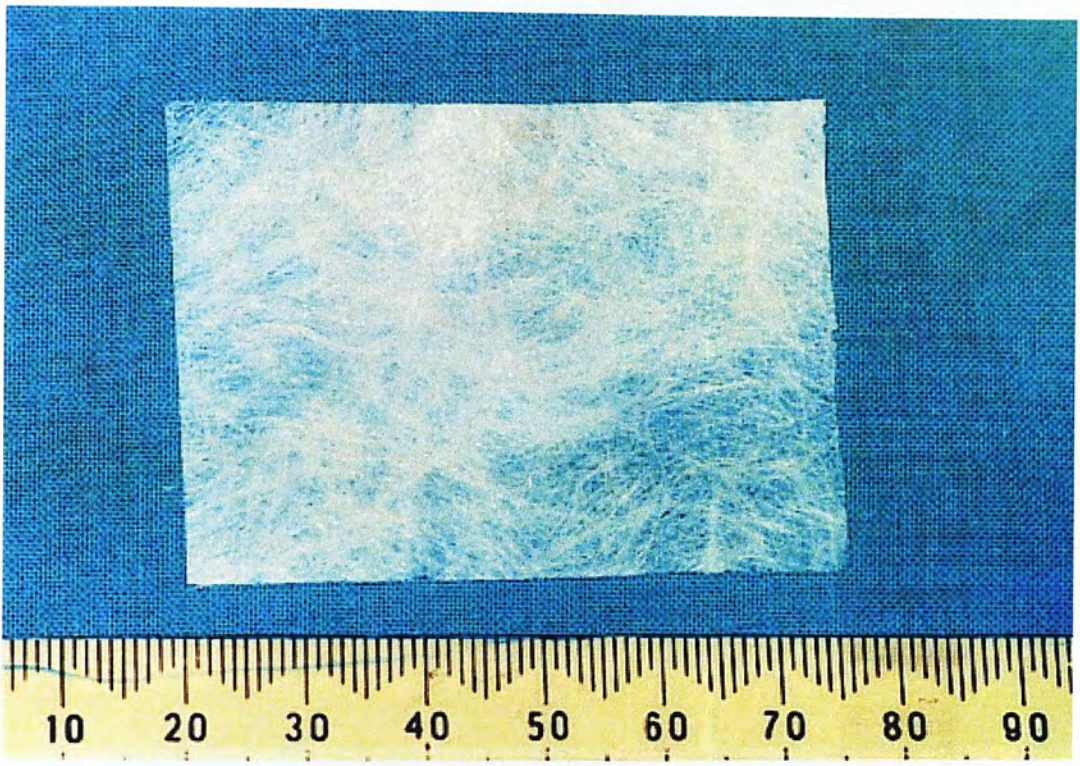


Fig 4 Picture to show the rectangle of glass polymer wrap material used for nerve entubulation.

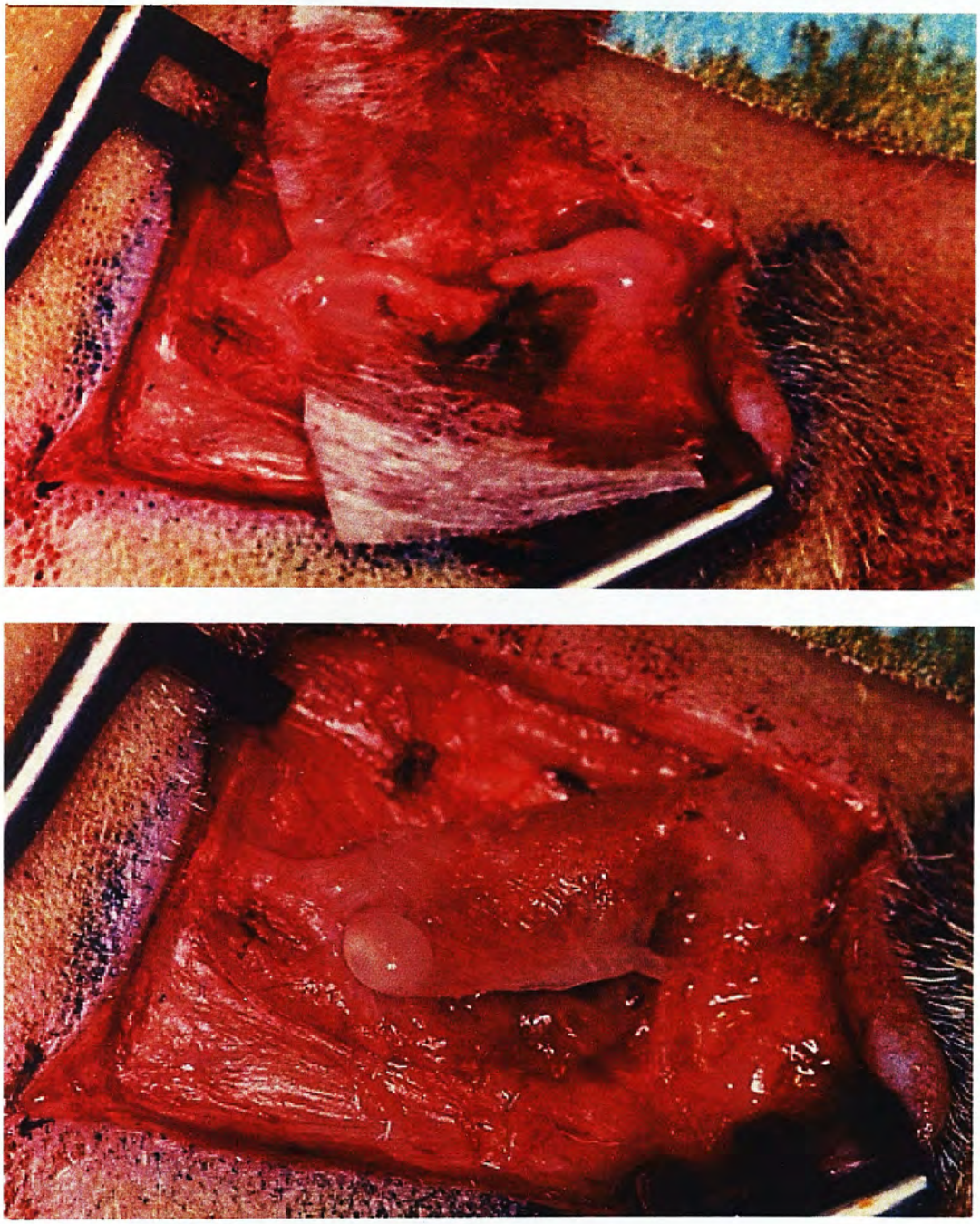


Fig 5 Pictures to show the transected median nerves ends wrapped in the CRG material. The first picture shows the transected nerve ends lying on the material. The bottom picture shows a fibrin glue blob to hold the median nerve end into the wrap formed by the material.

Group 6: Experimental repair – end-to-side neurorraphy

The median nerve was sharply transected as in the above control groups, 6cm from the ribcage. The proximal end of the nerve was turned back on itself and buried into neighbouring muscle to keep it well away from the end-to-side coaptation site. An epineurial longitudinal incision about 0.5 cm in length was made in the side of the adjacent ulnar nerve with a small sharp neurotomy knife with vision aided by the use of an operating microscope. The incision was recognized to be epineurial as intraneural vessels tended to bleed and the edges of the incision became obviously separate from underlying nerve coverings. The ulnar nerve had previously been dissected free from connective tissue taking care not to damage it.

Using interrupted 9-0 nylon sutures the cut distal end of the median nerve was sutured end-to-side to the window in the ulnar nerve using approximately 6 epineurial sutures. The posterior edge of the epineurial incision or window was sutured first taking care to make sure the sutures were knotted outside the epineurium. The middle posterior suture was place first then one above and one below this. The anterior wall was then sutured with three similar sutures, which were placed directly opposite the three posterior sutures as far as possible. Vision was aided throughout this procedure with an operating microscope. The position of the window in the ulnar nerve was measured first by holding the cut end of the distal portion of the median nerve against the ulnar nerve. This helped to ensure the neurorraphy was carried out without tension and also without too much slack in attached portion of the nerve.

Group 7: Experimental repair – double end-to-side neurorraphy

After exposure of the median and ulnar nerves as above the median nerve was again sharply transected with a knife 6cm from the ribcage. An epineurial window was made in the adjacent ulnar nerve with a neurotomy knife at the level at which the proximal stump had been and the transected median nerve could lie against it without tension. This proximal stump was sutured end-to-side to the window with 6 interrupted 9-0 nylon sutures. Another epineurial window was made 1cm distal to the first coaptation site on the ulnar nerve so as to allow the distal stump of the median nerve to lie against it with out tension. The distal stump of the median nerve was sutured end-to-side to the window again using epineurial sutures as above.

Wound closure

The wound was washed out with saline and closed with an absorbable 3-0 Vicryl (Ethicon) suture in a continuous fashion to the pectoralis muscle fascia and with an absorbable subcutaneous stitch to close the skin. Antibiotic (Tribiotic, Riker laboratories) and plastic film sprays (Nobecutane, Astra Pharmaceuticals ltd.) were applied.

Recovery

After the finish of the operation, the inhalational anaesthetic was gradually withdrawn until the animal was eventually only being ventilated with ten litres per minute of oxygen. When the animal started to take spontaneous breaths the endotracheal tube was removed. 50mg of an analgesic agent was also given intramuscularly at this time (Flunixin) and the animals that had undergone the glass wrap entubulation procedure received 1.5g of Cefuroxime (Zinacef, Glaxo,U.K.) to

minimize infection. When the animal was managing to ventilate adequately it was transferred to a trolley and taken into the recovery area adjacent to the theatre. The sheep were recovered in single clean pens with clean straw. They were propped lying on all four limbs by bales to help them get to their feet when they had recovered sufficiently to stand. They were checked every few minutes until they had stood up and were feeding. They were then left in these pens for 24 hours for further observation and then allowed back to join the rest of a flock. The operations lasted approximately 30 minutes each.

The sheep were left for 10 months in order for the nerves to regenerate. This has previously been shown to be an adequate amount of time for peripheral nerves such as the median nerve to regenerate sufficiently for muscle reinnervation (Fullarton et al. 2000).

During this time the animals were observed for the occurrence of wound infection and sores resulting from loss of sensation. Some of the sheep developed superficial wound infections. These responded to an intramuscular course for 5 days of 1.5g cefuroxime (Zinacef, Glaxo U.K.) The animals were checked every day during antibiotic treatment and were seen to remain well after the infection had cleared.

Assessment of recovery

The animals were brought back for the second experiment, which by Home Office legislation must be the terminal experiment, after a period of 10 months. Extensive assessment of recovery of the nerves distal to the sites of neurorraphy in each group of sheep was carried out. The emphasis of this was on reinnervation with recovery of function in target muscles.

Anaesthesia for assessment experiments

The animals were anaesthetized in the same way as for the preliminary experiments. Anaesthesia was induced and intubation took place prior to pressure-controlled ventilation with a combination of an inhalational anaesthetic agent (Halothane), oxygen and nitrous oxide. These experiments were acute and were therefore conducted with clean but not sterile technique. The procedures lasted about 3-4 hours each during which the animal's heart rate, oxygen saturations, temperature and signs of depth of anaesthesia was monitored as for the preliminary operations.

The right arm was shaved from rib cage to hoof and the area was cleaned with alcohol but not with antiseptic povidone iodine (Betadine) as for the sterile experiments. The limb was again stretched out and secured in a gutter type support with its volar surface facing upwards.

METHODS OF ASSESSING THE NERVE REPAIR

A number of tests were carried out to assess the repairs. These are detailed as follows:

Electrophysiological tests

Nerve conduction studies are a technical way of objectively assessing the state of function of the peripheral nervous system. The procedures of the studies are standardized to increase their reliability.

Recording an electrical impulse

A stimulus is defined as an external agent, state or change that is capable of influencing the activity of a cell, tissue or organism. In nerve conduction studies an electrical stimulus is generally applied to a nerve or muscle. In absolute terms the electrical stimulus is defined by duration in milliseconds, a waveform, and a strength or intensity measured in volts or ampères (current). The electric stimulus, in practical terms, has to be adequate to produce a motor or sensory response and can be graded as subthreshold, threshold, submaximal, maximal or supramaximal. A threshold stimulus is that which will produce a detectable response. A maximal stimulus is that which is of an intensity after which a further increase in its intensity causes no increase in the amplitude of the evoked potential. Supramaximal stimuli are used for nerve conduction studies and are of greater intensity than that used for maximal stimuli. By convention, an electrical stimulus of 30% greater current than that required for a maximal stimulus is used for supramaximal stimulation.

When a nerve is stimulated a reaction occurs somewhere along it. This reaction is monitored with recording electrodes, which can be surface or needle electrodes. Surface electrodes are non-invasive and record the electrical response of the nerve stimulation from the skin. They are usually metal discs of 0.5 to 1.0cm in diameter. Needle electrodes are invasive and record directly from nerve or muscle where they are placed. Direct recording is made by placing the electrodes over the nerve being stimulated. Indirect recording by placing the electrodes over a muscle can be used for motor conduction studies.

Stimulating peripheral nerves can occur by passing electrical current through the skin using surface disc electrodes as described above, or monopolar needle

electrodes that can stimulate the nerve directly. It should be noted that in practice stimulating the nerve percutaneously is difficult owing to accurate nerve localization. However needle electrode stimulation has the advantages of a) smaller stimulus intensities being required, b) the nerve can be stimulated more selectively and c) nerves that lie anatomically deep to surface structures can be stimulated.

With the recording electrodes placed over the muscle, a synchronized muscle contraction called a compound muscle action potential (CMAP) is recorded. The (active) recording electrode is placed over the motor point of the muscle, which is the point where a clear negative (upward) deflection is recorded when electrostimulation is applied to the nerve supplying that muscle. The reference electrode should be placed off the muscle on a nearby tendon or bone. The ground electrode is a metal plate that provides a large surface area of contact with the animal. It is usually larger than the recording and reference electrodes and is placed in between the stimulating electrode and the recording electrode.

Electrode set-up

In the experiments presented here, surface recording platinum disc electrodes of 0.5cm diameter and a bipolar platinum wire stimulating electrode was used for the maximum conduction velocity experiments (see picture below). For the jitter experiments percutaneous monopolar needle stimulating electrodes and a single fibre bipolar recording electrode were used. The ground electrode was a 2cm diameter flat metal disc.

All recording, reference, ground and stimulating electrodes including needle electrodes were cleaned after each use with soapy water and dried. Needle electrodes

were wiped clean with care. A thin film of electrode gel (Ten-20, Weaver and Co., Aurora USA) was used on each surface disc electrode to maximize conductivity. The electrode site on the skin of the sheep was cleaned with alcohol to make sure it was clean and free from oil to reduce impedance at the electrode/skin interface. All electrodes were fastened securely with surgical tape or a suture placed through the middle of the disc.

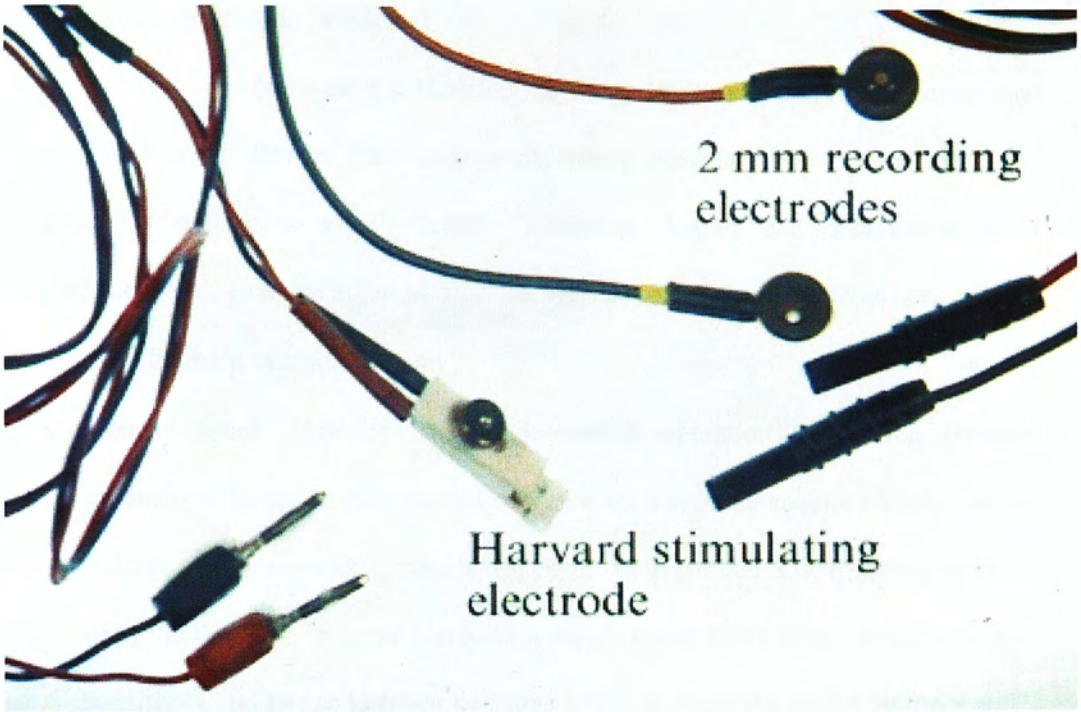


Fig 6 Figure to show the electrodes used in experiments and their distal connections.

The Synergy Mobile (Medelec)

The Medelec system ('Medelec', Synergy Multimedia, Oxford instruments) is a 2, 5 or 10 channel electromyograph, which provides facilities for EMG and evoked potentials to enable reliable recording, display and documentation of electrophysiological information from the nervous and muscular sytems.

The main components are:

- Control panel. This provides single control operation for setting essential parameters during an electrophysiological test such as the acquire switches which deliver a stimulus and acquire a response. An erase and a next switch to move onto the next site in nerve conduction velocity and EMG tests. It also contains sensitivity and sweep duration switches which changes the scales on the x and y axes.
- Monitor.
- Disk drives.
- Power supply unit. The system has a 1250W power supply unit.
- Input/output connections.
- Pre-Amplifier. This receives signals from the recording electrodes. The two-channel pre-amplifier has 2 positive, 2 negative and 1 neutral (earth) 1.5mm touch-proof connections. The impedance check button when pressed shows impedance levels.
- Keyboard and Mouse.
- Stimulator probes. This system has a dual external stimulator capacity. The two probes were marked 'A' and 'B' with a permanent marker. They are hand-held devices that can be used to stimulate nerves through the skin using the two

stimulator pins. The pin electrodes can be removed to allow access to two 1.5mm touch-proof connectors (cathode and anode ports) that can be connected to wires with disc electrodes attached for use as a static stimulating device. This system was used in our experiments.

- Trolley. The Medelec is mounted on a trolley, which is equipped with a mounting arm for the amplifier. This provides flexible and height-adjustable mounting for the pre-amplifier and electrical hand-held stimulators.

Fig 7 Picture to show the Medelec machine. The control panel can be seen on the right side of the picture mounted on the arm. The stimulating and recording electrodes are connected here and the hand-held stimulators can be stored just to the left side of the panel.

From the test manager on the right of the entry screen the test to be performed was selected from the appropriate folder. From the test menu window, the folder containing the test to be performed is 'clicked' with the mouse.

Transcutaneous stimulated jitter (TSJ)

Theory

Electromyography is a widely used method of assessing motor units (motor neuron, its axon, and the muscle fibres innervated by its axon), in the study of neuromuscular disorders. The method involves recording the motor unit action Potentials (MUAPs), which are set up when voluntary or stimulated contraction the muscle takes place, using a needle electrode inserted into the muscle. The MUAP is the sum of the electrical activity of the skeletal muscle fibres in a motor unit that are within the recording range of the electrode and gives a measure of the degree of innervation of the muscle. This makes it useful in neuromuscular disorders and nerve repair (Stålberg 1966).

Single fibre electromyography (SFEMG) is a technique for selectively recording individual muscle fibre action potentials using a concentric needle electrode (Gath & Stålberg 1979). The needle electrode has a small recording surface (25 micrometre diameter), which is satisfactory for recording single fibre action potentials. The diameter of these muscle fibres is normally 50-80 micrometres. The electrode has a 0.5mm steel cannula containing an insulated silver wire. The steel casing forms the anode and the negative pole of the electrode is an opening 3-5mm from the tip of the electrode, which exposes an uninsulated portion of the silver wire. This recording surface faces away from the bevel of the needle to avoid recording action potentials

from mechanically damaged muscle fibres during needle insertions. This type of needle helps selective recording of individual muscle fibres from the muscle under study due to its smaller pickup area. This smaller area causes less distortion of the electrical field that surrounds a muscle fibre. The oscilloscope shows sharp peaks with minimal interference and distortion.

A single fibre action potential is recorded from a single muscle fibre when the nerve supplying that muscle is stimulated. It has duration of 1ms and amplitude of usually less than 5mV but can be as high as 25mV. It is more reliable to identify single fibre action potentials on the oscilloscope screen by their shape rather than their amplitude as the latter decreases with increasing distance of the tip of the recording electrode from the optimal stimulating site on the muscle fibre and the former remains constant with successive stimuli. The optimal site is found by twisting the needle electrode around in the muscle and noting a well-defined action potential with an almost 90° take-off. In practice, single fibre electrode only records from one muscle fibre in about half of all recordings as in a normal motor unit fibre are only separated from one another by a few hundred microns. No measurements should be taken until clean single fibre potentials are obtained. Sometimes, on the oscilloscope screen, two or more action potential spikes may be seen. These are from different muscle fibres. The interpotential interval (IPI) between the action potentials is due to the difference in conduction time from the last common nerve branching site, through the neuromuscular junction to the recording site on the muscle fibre. It is usually about 0-4 ms in duration. Longer interpotential intervals may indicate aberrant motor end-plate positions or slow conduction along a nerve or muscle fibre. For each action potential pair, the interpotential interval varies. This is called 'jitter' (Dorfman 1984).

The variability in the IPI probably results from fluctuations in the time it takes for motor end plate potentials at the neuromuscular junction to reach the threshold for further progression of the impulse.

When a peripheral nerve is cut the neuromuscular junction of the muscle it supplies degenerates within 48 hours after denervation (Birch 1986). If the nerve is repaired correctly reinnervation will occur from regenerating motor axonal sprouts growing down from the proximal nerve stump. When these axons reach their target muscles, new end plates begin to develop and establish new neuromuscular junctions (Hakelius, Nystrom, & Stålberg 1975). Immature neuromuscular junctions show increased variability in the time of neuromuscular transmission owing to differences in the threshold potentials seen at the end plate (Stålberg, Ekstedt, & Broman 1971; Trontelj, Stålberg, & Mihelin 1990). However as the neuromuscular junctions mature the end plate potential becomes more stable (Wiechers 1990). It is this stability that jitter can measure in order to assess the timing and quality of repair. The variability in the transmission of the electrical impulse down the muscle fibre has been shown to be minimal in normal muscle (Trontelj & Stålberg 1983).

Stålberg first measured jitter in 1964 to assess patients with diseases that affected the neuromuscular junctions such as motor neurone disease and multiple sclerosis. He found the technique difficult to perform at first, as it required patient co-operation and needed two (MUAPs) to occur at the same time within the scope of the oscilloscope sweep speed (Stålberg1966). Trontelj developed a method of measuring jitter without the need of patient co-operation or two simultaneous MUAP's subsequently in 1986. It was called stimulated jitter as it involved stimulating the terminal motor axons directly and recording the associated MUAP. This method was

much more straightforward to perform and became popular with as a clinical test (Trontelj et al. 1986; Trontelj & Stålberg 1992). Degeneration and reinnervation associated with motor neurone disease are similar to these processes occurring when a nerve is cut and therefore, the measurement of jitter to assess nerve regeneration after injury seems useful (Trontelj & Stålberg 1995).

Given the theory behind the 'jitter' measurement and its application, it is apparent that this measurement is useful for assessing muscle reinnervation in these experiments. In each of the experiments the median nerve is transected and repaired in a different way in each experiment. For each of the experiments the muscle used to test 'jitter' was the flexor carpi radialis muscle as this is known to be innervated exclusively by the median nerve.

Method to assess TSJ in the sheep

After the designated time of 10 months each of the experimental sheep were brought back to theatre for assessment of their nerve repairs.

Each animal underwent general anaesthesia as the previous time and was placed on their backs on the operating table. The experimental limb was stretched and secured so the volar surface was facing upward. The skin of the forearm of the experimental sheep was shaved right down to the hoof area and the skin over the FCR muscle was cleaned with chlorhexidine. Two monopolar needle electrodes were used to stimulate this muscle. The cathode was inserted through the skin into the FCR muscle at the motor point (where the nerve enters the muscle) and the anode is placed 0.5cm proximal and lateral to the cathode. The stimulus pulses of current were delivered to the electrodes with duration of 50μs and a frequency of 5 cycles per

second by the Medelec machine. The current was increased by 0.1mA increments until the muscle was seen to twitch rapidly. Usually less than 5mA was adequate.

Action potentials from distant muscle fibres have a higher proportion of low frequency components than those from adjacent muscle fibres. In order to avoid 'noise' interfering with the recording of the MAP therefore increasing the selectivity of the recording, the frequency of the high pass filter was increased to 500 Hz. This removed recordings of more distant fibres. This happens because muscle and connective tissue normally act as low pass (high cut-off) filters. The low-pass filter was set to 10kHz to preserve high-frequency components but reduce interference. High frequency (low pass) filters consist of a resistor (in series) and capacitor (in parallel). Initially all current flows through the resistor, then as the capacitor charges progressively, less current through resistor which in turn slows charging of the capacitor and therefore takes longer for output voltage to equal input voltage.

If a slow sine wave is applied to the system the capacitor has time to charge, but if a fast wave is applied, there less time available for capacitor charging and there is minimal output at high frequencies. The cut off point of a filter is commonly defined as output 70% of input (-3dB).

Low frequency (high pass) filters have the capacitor in series and the resistor in parallel and are therefore reversed compared to high frequency. Output voltage must equal input voltage, so the potential difference across resistor equals input potential difference minus the potential difference across the capacitor. In slow i.e. low frequency current, the capacitor has time to charge so there is a small potential difference across the resistor and output is blocked. High frequency current does not

have time to charge capacitor so there is a high potential difference across resistor and high frequency current gets through.

These filters are part of the Medelec system and can be manually set. The signal was displayed on the Medelec monitor. The action potential is triggered by the stimulus artefact and the sweep speed was set to 1ms/div.

The SFEMG electrode was inserted into a twitching portion of the muscle approximately 2cm distal to the cathode. The train of stimuli from the Medelec causing the muscle to twitch was connected to a loud speaker within the machine, so when the needle moved close to a contracting muscle fibre the pitch of the sound from the Medelec changed. The position of the electrode was adjusted by rotating and advancing it until the recording of the muscle action potential was satisfactory and remained steady. The electrode was never retreated so as to avoid recordings from mechanically damaged muscle fibres during needle insertion. With the electrode in position showing a good trace of the muscle action potential, a recording of this was taken after 50 stimuli of the terminal motor axon. Stålberg showed that this allowed for a statistical sample of that motor unit to be produced (Stålberg1966).

The latency between the stimulus pulse and the muscle fibre action potential is called the inter-potential interval (IPI) and was measured for each stimulus.

This recording of the action potential for 50 stimuli was repeated at 20 different sites in the muscle and an average of these values was recorded as the 'average jitter' for that muscle.

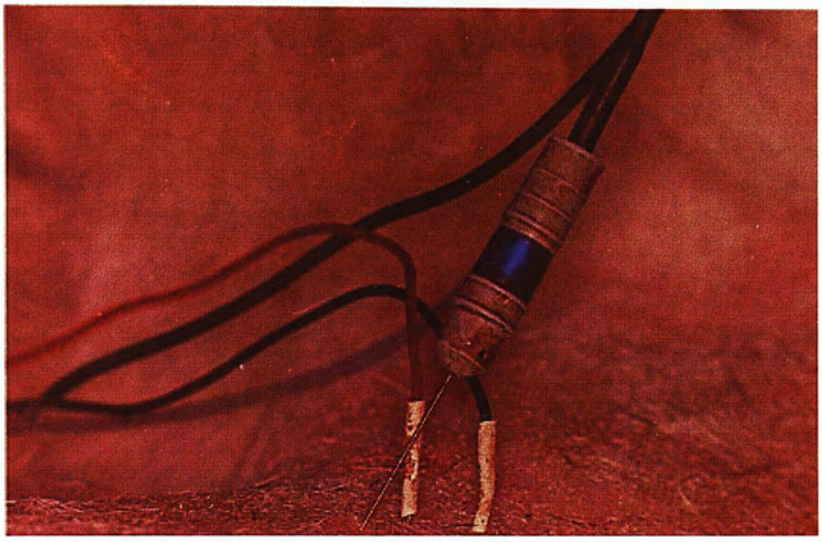


Fig 8 Picture to show the measurement of 'jitter'. The monopolar stimulating electrodes are red and black to denote the anode and cathode and the recording electrode is placed in the twitching part of the muscle.

The variability between the IPIs is the *jitter* value or mean consecutive difference (MCD) and is calculated using the following equation:

$$MCD = \sum \left(\frac{IPI_n - IPI_{n-1}}{n} \right)$$

Where:

MCD = mean consecutive difference (μs)

IPI = interpotential interval (ms)

n = number of stimuli

Aims of TSJ study

The 'jitter' value gives us a measure of the maturity of regeneration of the nerve innervating the muscle under test. Therefore it was important to measure the average jitter value in the FCR muscles for all the groups of sheep in order to compare normal values to the experimental values. It was also important to assess the muscles supplied by the donor nerve (ulnar nerve in these experiments), to find out if there has been any permanent damage caused to it during end-to-side neurorraphy.

Maximum conduction velocity

Theory

The maximum conduction velocity of a nerve represents the velocity of the fastest conducting axons. In a motor nerve this is only 5% of the total population of

motor neurons (Dorfman, Cummins, & Abraham 1982). Despite this, the maximum conduction velocity gives an objective, non-invasive and reliable idea of nerve function. This partly because disease processes affect all nerve fibres uniformly or predominantly the faster fibres. The results from measuring the maximum conduction velocity have proved to be reproducible, it is easy to perform and does not destroy sample tissue. The maximum conduction velocity is extremely useful in reviewing the progress of regeneration after surgical nerve repair and to assess nerve lesions.

One of the first studies that recognized altered nerve conduction in human disease was by Hodes et al in 1948. He reported that conduction velocity is slowed in regenerating nerve (Hodes, Larrabee, & German 1948). The Second World War brought about some pioneering experiments, in which the researchers observed the effects of stimulation of denervated muscle, but it was not until after this time that a place was established for nerve conduction studies (Seddon 1954).

The nerve behaves like an electrical cable that has a central core conductor surrounded by an insulator. An unmyelinated nerve fibre axon consists of a phospholipid semi-permeable membrane surrounding a long cylinder of amorphous gel matrix. This gel or cytoplasm has a viscosity five times that of water and is a poor conductor compared to a metal like copper. Current will travel less than 1mm along the axon before being dissipated by the internal resistance of the axon cytoplasm and thorough the uninsulated membrane. Conduction of a nerve impulse depends on movement of polarized ions across the semi-permeable membrane of the axon. The membrane acts as a high-resistance pathway, while both the environment outside the axon and the axoplasm are low-resistance pathways. This configuration allows ions to move parallel to the inside and outside of the membrane with relative ease. Membrane

potential decreases exponentially, but the distance current can flow down an axon is mainly due to the internal resistance of the axon. The internal resistance of the axon becomes less as the fibre diameter increases which means that in larger diameter fibres an electrical signal can travel further.

The biphospholipid structure of the membrane has the capacity to store charge (capacitor), and the nerve's conduction properties vary with its resistance and capacitance. Explaining nervous signal propagation was first proposed in the early twentieth century by Hermann, after it was originally derived by Lord Kelvin for the transatlantic telegraph cable (Hermann 1905).

Conduction velocity is affected by many different factors both anatomical and physiological. Anatomical factors affecting the nerve include axon and fibre diameter, myelin sheath thickness, Schwann cell structure and internodal length. Physiological factors include temperature and age of the patient and the biochemical environment surrounding the nerve. Conduction velocity slows considerably and action potential waveform configuration may change if the nerve is cool (<30°C). This can lead to falsely positive tests and may suggest abnormalities that are not actually present. In the early fifties it was demonstrated that in humans over 60 years of age the maximum condcution velocity can be decreased by as much as 10% (Norris, Shock & Wagman 1953). Part of the reasons for this, however, were thought to be due to calloused or wrinkled skin, oedema, excess perspiration, and the patient's inability to relax or control the extremity or hand thereby maing a study more technically difficult (Carter et al. 2000; Johnson & Olsen 1960).

The larger the diameter of the nerve the faster the conduction velocity. This was observed by Gothlin in 1907 with several scientists confirming these results in

later years e.g. Gasser and Erlanger in 1927 (Gasser & Erlanger 1927) and Matthews in 1929 (Matthews 1929). Further work found the conduction velocity of a nerve to be proportional to the square of its diameter (Erlanger & Gasser 1937). The resistance and capacitance of a nerve are expressed in terms of a unit length of nerve, which does not give any indication of how these measurements vary with diameter of the axon. A period of time is required for the membrane to 'charge', which means that there is a delay in the change of the membrane potential after an applied current. The longer this period of time, the longer the membrane takes to depolarize and the slower the spread of current and *vice versa*. The speed at which the membrane responds to electrical signals is unrelated to nerve fibre size but the distance a signal spreads is the square root of the nerve fibre diameter (Dorfman 1984; Gelberman 1991a).

Myelin significantly increases conduction velocity by causing current to flow down the centre of the axon and exit only at the nodes of Ranvier. Myelin provides the axon with a high radial resistance. Conductance of the axon is the reciprocal of resistance so the high radial resistance decreases the time the membrane takes to store charge (capacitance) and therefore increases the conduction velocity. The action potential jumps between nodes, which allows a small diameter axon to conduct as rapidly as a large unmyelinated fibre. Several researchers have demonstrated the relationship between myelin sheath thickness and conduction velocity (Bergland 1960; Sanders & Whitteridge 1946), however, variations in the thickness of the myelin sheath, between fibres of the same size in the one nerve and along the course of an individual nerve fibre still lying in the nerve trunk, have not been taken into account. Computer modelling of conduction in a nerve has allowed researchers to look at the effects of altering the axon and fibre diameter and the myelin sheath thickness in more

detail (Waxman 1980). These studies have shown that myelinated fibres conduct faster than ummyelinated fibres down to an axon diameter of 0.2μm, when it had originally been thought that thin myelinated axons would conduct slower than thicker unmyelinated axons due to the affect of the axon diameter.

The relationship between the axon diameter and the myelin sheath thickness is such that if the myelin sheath thickness is increased with a fixed axon diameter, the advantage to the conduction velocity will be offset by the increase in axial resistance. It was predicted that the optimal ratio of axon diameter to fibre diameter should be 0.6. This is called the 'G'ratio (Rushton 1951).

$$\textbf{G-ratio} = (d) / (D)$$

Where: (d) = axon diameter

And (D) = fibre diameter

The Schwann cells that make peripheral myelin are extremely sensitive to ischaemic or mechanical injury. Conduction failure can result in myelinated nerves if the myelin is interrupted or a local anaesthetic disrupts excitation over several nodes (Caruso, Labianca & Ferrannini 1973). It is known that after peripheral nerve transection the maximum conduction velocity only returns to 80% of its normal level (Berry, Grundfest, & Hinsey 1944).

In his classical experiments Lillie described the speed of transmission in a piece of metal wire enclosed by an interrupted tube made of an insulating material to be greater than in one enclosed by a continuous tube (Lillie 1925). This suggested a relationship between internodal length and conduction velocity. Internodal length varies depending on the thickness and length of the nerve fibre. In general, the length

increases linearly with the diameter of the fibre. Internodal length may also vary in fibres of the same diameter while successive nodes in the same fibre may have different lengths (Schlaepfer & Myers 1973). The number of internodal segments remains constant throughout development and their length therefore increases as the nerve fibre elongates during growth. The internodal length will be greater where elongation occurs rapidly over long distances. The remyelination of regenerated nerve fibres in the adult produces nodes that are short and of about the same length for all fibres regardless of their calibre (Jacobs & Cavanagh 1969).

It was demonstrated in 1934 that segmentation of the nerve fibre influences its conduction properties (Erlanger & Blair 1934). However in other work it had been demonstrated that, in regenerating nerve fibres where the internodal length is constant, there was no relationship between internodal length and conduction but that the latter was directly related to myelin thickness (Coppin & Jack 1972; Sanders & Whitteridge 1946). A study in 1978 used computer simulation of conduction in myelinated fibres to examine the dependence of conduction velocity and spike configuration on internode distance. It showed that for nerve fibres with very short internodal distances, small local changes in these distances should substantially affect the conduction velocity (Brill et al. 1977).

Measuring the conduction velocity

To measure the nerve conduction velocity, percutaneous depolarizing current is delivered over a peripheral motor, sensory, or mixed nerve and a compound motor or sensory nerve-action potential (CMAP/SNAP) at a measured distance away from the stimulus through recording electrodes placed over the muscle belly or nerve is

recorded. The nerve is stimulated at 2 or more points along its course, with the anode 2 to 3 cm proximal to the cathode. A depolarized nerve carries current in both directions (ortho-and antidromically), so the elicited responses can be recorded distally (Carter et al. 2000). In the present experiments the motor responses of the FCR and FCU muscles in order to calculate the conduction velocities were used.

Surface recording electrodes, in general, are better than needle electrodes for recording a compound muscle action potential as they assess contributions from all discharging units. The onset latency of the CMAP indicates the conduction time of the fastest fibres and its amplitude is proportional to the number of available axons. Needle electrodes register only a small portion of the MAP but are useful in measuring CMAPs from small atrophic muscles as there is less interference from neighbouring electrical discharges and the onset of the MAP is sharper.

The recording cathode is placed on the motor point of the muscle (which is described in the previous section) innervated by the nerve under test, and the recording anode (a reference electrode) is placed usually on the tendon of the muscle. Depolarization under the cathode results in the generation of a nerve action potential, whereas hyperpolarization under the anode tends to block propagation of the nerve impulse. The pulses of moderate intensity are used to adjust the position of the cathode until there is no further change in size of the muscle potential. The stimulus given is always increased until it is supramaximal, this allows consistency so that serial studies can be compared. The use of a 20 to 30% supramaximal intensity guarantees the activation of all the nerve axons innervating the recorded muscle. With the electrodes in the positions described, the propagating muscle action potential gives rise to a simple biphasic waveform with initial negativity. A small positive potential may

precede the negative peak with inappropriate positioning of the recording electrodes (Kimura 1989b).

The latency of the waveform consists of two components: 1) nerve conduction time, from the stimulus point to the nerve terminal, and 2) neuromuscular transmission time, from the axonal terminal to the motor end plate, including the time required for generation of the muscle action potential. Onset latency is a measure of the fastest fibres (Kimura 1989b). To measure the motor nerve conduction time, the time for neuromuscular junction (NMJ) transmission and generation of the MAP must be subtracted.

The nerve was stimulated at two different points along it using two separate electrodes (S1 and S2). The distance between the electrodes was kept as far as possible the same for all the experiments. For the experimental groups S1 and S2 represent sites proximal (S1) and distal (S2) to the repair sites. The nerve was stimulated at both S1 and S2 separately producing two CMAPs, M1 from S1 stimulus and M2 from S2 stimulus. The latency difference between these two CMAPs effectively removes the time for NMJ transmission and generation of the MAP common to both stimuli. It therefore represents the time required for the nerve impulse to travel between the two electrodes. The conduction velocity is derived as the ratio between the distance from one point of stimulation to the next and the corresponding latency difference.

CV_{max} = **distance *S1* to *S2* / (latency *M1*- latency *M2*)**

Where: CV_{max} = maximum conduction velocity in m s^{-1}

S1 = proximal stimulating electrode

S2 = distal stimulating electrode

M1 = Compound muscle action potential after stimulating S1

M2 = Compound muscle action potential after stimulating S2

Practical aspects of measuring the conduction velocity on the Medelec

The impedance of the electrodes was checked by pressing a key on the control panel. The impedance should be 8 kΩ and if this correct a green LED lights up. Practically the conduction velocity between the two stimulating electrodes S1 and S2 was measured using the software on the Medelec, from which the stimuli were being delivered and data are acquired and stored in the memory and displayed on its monitor. This occurs when Acquire is ON. The stimulus intensity was set to 0. The stimulus electrodes were checked to make sure they had good connection with ports of the remote 'stimulator A'. The Acquire ON key was pressed to stimulate and acquire responses. Using the 'stim intensity' rotary control to the intensity of the electrical stimulus was gradually increased until there was no increase in amplitude of the displayed CMAP trace on the screen (supramaximal stimulus). When an acceptable trace with a clear take-off point was seen the Acquire key was pressed again to switch it off. The traces were averaged by setting the 'averager on/off' to 'on'. The next key was pressed to record a new trace below the previous one, this previous one being stored in the active store facility of the software of the machine.

To calculate the velocity, the distance between S1 and S2 was measured with a ruler and entered into the active distance field on the display on the screen using the keyboard, the 'enter' was pressed to accept the distance and to calculate the conduction velocity.

Method

For each group of sheep the maximum conduction velocity was measured as an indicator of nerve recovery. For the end-to-side group the conduction velocity was measured from the ulnar nerve proximal to the neurorraphy site to the median nerve distal to the neurorraphy site. The conduction velocity of the donor (ulnar) nerve was also measured, proximal and distal to the neurorraphy site. This was to assess the extent of any damage caused to the donor nerve.

For the double end-to-side group four sets of conduction velocities were measured. Each conduction velocity was measured proximal and distal to the two neurorraphy sites and were set out as follows:

Donor nerve (ulnar to ulnar) U→U

Attached nerve (median to median) M→M

Ulnar to median U→M

Median to ulnar M→U

It is thought that these measurements will help to build up a picture of the patterns of reinnervation of the attached nerve. The recording electrodes were placed to record from the FCR for the attached nerve and the ulnar to median pathways and from the FCU for the donor nerve and median to ulnar pathways. The attachment of these skin-recording electrodes around these muscles is as previously described above.

Exposure

An incision was made from the sternum longitudinally over the surface of the volar humeral area to the elbow in line with the superficial pectoral muscle which is seen to 'tent' when the limb in stretched out during set up of the animal on the operating table. The length of the incision was about 20cm, which allowed full exposure of the old operation site including the full lengths of the ulnar and median nerves in this area. There was a lot of scar tissue around the neurorraphy sites, but by palpation of the brachial arterial pulse the neurorraphy sites could be fairly easily identified. The ulnar and median nerves lay just next to this. There was usually a reddish bulbous swelling around the neurorraphy sites as well which helped in identifying the position of the nerves. This swelling was likely to represent a neuroma. The proximal segments of the ulnar and median nerves were dissected out bluntly using dissecting scissors. In order to distinguish between the ulnar and median nerves, a stimulating electrode was applied in turn to each nerve. Surface electrodes were placed on the target muscle, the cathode on the motor point of the FCR muscle and the anode on a tendon distal to the FCR muscle belly. When the ulnar nerve was stimulated rather than the median nerve a very distinctive CMAP of the FCU muscle was displayed on the Medelec oscilloscope screen compared to the CMAP of the FCR. This is because the ulnar nerve supplies more than one muscle in this region of the sheep's upper limb, giving a distinctive triple peaked CMAP. The main muscle being the FCU that is about four times the size of the FCR, and in some animals was seen to be bipennate. This occurs even if the recording electrodes are placed on the FCR muscle owing to transmitted MAPs from the surrounding contracting muscles supplied by the ulnar nerve.

The median nerve only supplies the FCR in this region giving a characteristic smooth single peaked CMAP. The transmitted MAPs from the contracting muscles supplied by the ulnar proved to be a particular problem when trying to record the CV_{max} from the ulnar to median nerve end-to-side and double end-to-side pathways. In these experiments the ulnar nerve had to be transected proximal to the site of neurorraphy to abolish surrounding muscle contraction. In fact, to make sure the FCR CMAP was not contaminated by transmitted MAPs from surrounding muscles, the ulnar nerve was transected in all of the assessment experiments. In the two end-to-side groups the segment of the nerve distal to the site of neurorraphy was transected.

Practical aspects of measurement

When identification of the nerves was complete, the conduction velocity was measured across the sites of neurorraphy for each group. For the *epineurial suture, glass wrap* and *autologous graft* groups this was measured on the median nerve. For the end-to-side group, the conduction velocity was measured across the ulnar nerve to test for possible damage during end-to-side nerve attachment. This was then transected in order to achieve a pure FCR CMAP when the conduction velocity was measured across the ulnar to median nerve end-to-side neurorraphy. For the double end-to-side group, the ulnar nerve conduction velocity was measured across the double neurorraphy site to test for donor nerve damage and from the median nerve proximal to the site of neurorraphy to the ulnar nerve distal to the site of neurorraphy. The ulnar nerve was then transected distal to the site of neurorraphy, again in order to achieve a pure CMAP from contraction of the FCR.

After careful dissection of the proximal and distal segments of the median and ulnar nerves, the conduction velocity of each nerve was measured taking each one in

turn. A low impedance bipolar platinum stimulating electrode was placed under the nerve 2cm proximal to the site of neurorraphy with the cathode lying distally, S1. A similar second bipolar stimulating electrode was placed under the nerve distal to the site of neurorraphy, S2. The electrodes were made so that they were set into a plastic casing to keep them clear from the bed of the wound. This was to prevent current spreading from the stimulating electrodes and activating the nerve at a different point or activating contracting of surrounding muscle. One problem with this type of electrode was that the gap between the two wires of the electrode had to be kept dry at all times to avoid shorting out of the current, but the nerve had to be kept moist. Before each nerve stimulation took place therefore, the nerve was sprayed with saline and the gap between the electrodes was dried with a cotton bud.

S1 was connected to 'stimulator B' on the Medelec machine and S2 was connected to 'stimulator A'. The preparation was kept moist at regular intervals with saline and a unipolar metal disc electrode connected to earth was attached to the right lateral chest wall. The surface disc recording electrodes were placed on the FCU or FCR muscles depending on which nerve or pathway was being tested. The recording cathode was placed over the motor point (as described before) and the reference electrode distally over the tendon. For the end-to-side group the conduction velocity across the ulnar to attached median nerve pathway was being tested the so the CMAP was recorded from the FCR.

A short supramaximal stimulus was applied from the signal generator of the Medelec machine. Direct current stimulation was used and the size of the supramaximal stimulus varied from 2mA up to 100mA depending on the nature of the repair being tested. 50μs stimulus was used so that the length of the stimulus was

insignificant compared with that of the action potential. The output from the stimulators was connected to the Medelec and the CMAPs were recorded on the monitor. A sweep time of 2 or 5ms per division was used and filters were set with a wide bandwidth (50 to 50,000Hz) in order not to alter the waveform and hence measurements. These were set on the Medelec machine. The polarity was adjusted so that the first peak of the CMAP was positive. The first object of this procedure was to establish the presence or absence of an action potential across the neurorraphy sites. The next objective was to obtain a conduction velocity for the fastest fibres. The signal averaging facility of the Medelec was used to average sixteen action potentials in order to increase the signal to noise ratio. The S1 and S2 CMAP traces, M1 and M2,were recorded on the monitor of the Medelec machine separately and the take-off points on each trace marked electronically. The distance between the two electrodes was measured using a ruler. The conduction velocity was calculated using the equation previously discussed.

Abnormalities of the conduction velocity

Axonal damage or dysfunction results in loss of amplitude, whereas demyelination leads to prolongation of conduction time. There are three basic patterns of abnormalities that characterize motor nerve conduction studies when the nerve proximal to a lesion is stimulated.

1) Reduced amplitude with normal or slightly increased latency.
2) Increased latency with relatively normal amplitude.
3) An absent response.

In the first pattern, a stimulus below the site of nerve injury may still produce a normal CMAP, even though proximal stimulation above the site of injury has a reduced amplitude. This does not differentiate between a lesion such as neurapraxia or early axonotmesis in the first few days as degeneration may take up to five to seven days to fully develop. After this time the degenerating axons have lost their excitability. In the second pattern of response of the injured nerve to stimulation, slowed conduction is associated with a relatively normal amplitude when the nerve is stimulated above the lesion. This implies segmental demyelination affecting a majority of the nerve fibres.

An absent response indicates that a majority of nerve fibres are not conducting across the site of the injury or repair. Axonotmesis, neurapractic injury and neurotmesis can be distinguished from each other. In all types of injury nerve stimulation distal to the site of injury elicits a normal CMAP for the first 5-7 days. In axonotmesis and neurapraxia proximal stimulation results in a small response from distal muscles. After this time, with neurotmesis, stimulation below the site of injury produces no CMAP, because the neuromuscular junction is degenerate owing to loss of axon continuity. Loss of nerve excitability follows Wallerian degeneration. In axonotmesis, consecutive CMAPs show a progressive decline in amplitude over the first 5 days and degeneration occurs in all motor fibres by day 5 to 7 after injury and is complete by day 7 to 9 (Chaudhry & Cornblath 1992). In a purely neurapractic injury the distal CMAP is always present and does not decrease in amplitude, unless there is disuse atrophy. This is because no axon loss or Wallerian degeneration occurs and the distal nerve segment remains normally excitable (Carter et al. 2000).

Two to three weeks after axonot- or neurotmesis, needle EMG demonstrates fibrillation but no real action potentials and positive sharp waves (Kraft 1990). The time between injury and onset of fibrillation potentials partly depends on how proximal the lesion is to the spinal cord. These potentials decrease in size and number as reinnervation occurs. Recovery depends solely on axonal regeneration, which may or may not occur depending on the degree of injury to the nerve. The earliest evidence of axonal regrowth and establishment of new neuromuscular junctions after neurotmesis is the presence of small, long duration, unstable, motor unit potentials on needle EMG. This usually precedes the onset of clinically evident voluntary movement (Dorfman 1990).

Aims of maximum conduction velocity studies

The maximum conduction velocity is an accepted, easily repeatable and reliable way of assessing the recovery of function after nerve repair. It is important to compare normal values to values obtained during conventional nerve repair as well as to those obtained in the end-to-side experiments. It is also interesting to assess the donor nerve conduction properties for signs of damage during the end-to-side neurorraphy procedures.

F wave measurement

Theory

DEFINITION: The F wave is a compound action potential evoked intermittently from a muscle by a supramaximal antidromic electrical stimulation of a motor nerve.

Compared with the maximal amplitude M wave of the same muscle, the F wave is smaller, has variable configuration, and a longer more variable latency with disatl sites of stimulation (DeLisa et al. 1994).

Conventional nerve conduction studies do not usually contribute to the investigation of more proximal lesions. Measurement of the F wave helps in assessing motor conduction along the most proximal segment, because it results from the backfiring of antidromically activated anterior horn cells. The inherent variability of the latency and configuration makes the use of F wave less precise than that of direct compound muscle action potential. A supramaximal electric shock delivered to a nerve often elicits a late response in innervated muscle. Since the original description authors have debated its neural source. The F wave occurs after the direct motor potential or M response. With more proximal stimulation, the latency of the M response increases, whereas that of the F wave decreases. The impulse that elicits the F wave first travels away from the recording electrodes toward the spinal cord before it returns to activate distal muscles. The F wave is not really a true reflex as the afferent and efferent arc of this response consist of the same alpha motor neuron.

Motor neurons subject to recurrent stimulation only fire infrequently. Recurrent discharges develop in only a limited number of motor units, in part because the antidromic impulse fails to enter the somata in some of the motor neurons. This type of block often occurs in the axon hillock where membranes change and more frequently in the smaller, lower threshold motor neurons, which rapidly depolarize. The incidences of the F wave, may in theory, favour the larger motor neurons with the faster conducting axons. This provides a reason for using the

minimal latency of the F wave as a measure of the fastest conducting fibres. The F wave is elicited in approximately 1 to 5% of antidromically-activated motor neurons regardless of their peripheral excitability or conduction characteristics (Kimura 1989c).

The amplitude and frequency of the F wave provide a measure of motor neuron excitability, but the relationship is physiologically complex. The amplitude of the F wave increases in disorders of the lower motor neuron, presumably because regenerated axons supply an increased number of muscle fibres.

In the presence of collateral sprouting in the proximal portion of the nerve, a submaximal stimulus excites one branch of the axon but not the other, the antidromic impulse up to the point of branching and turns round to proceed distally along the second branch. Electrical impulses of higher intensity, activating both branches distally, eliminate the response, because two antidromic impulses collide and cancel each other out as they turn round at the branching point. Therefore no late response in the muscle is seen.

Measurement of the F wave response

A supramaximal stimulus applied at practically any point along the course of a nerve elicits the F wave. Placing the stimulating anode distal to the stimulating cathode or off the nerve trunk avoids anodal block of the antidromic impulse.

A bipolar platinum stimulating electrode encased in a plastic casing (as before) was placed under the nerve under test. In the normal ulnar and median nerve groups the electrode was place under the corresponding nerve for that group. For the end-to-side groups the electrode was placed on the median nerve distal to the

neurorraphy site. A surface electrode placed over the motor point of the FCR muscle served as the active electrode against the reference electrode, which was placed over the tendon. An earth electrode was placed away from the recording site on a shaved part of the sheep's ribcage. The stimulating electrode was connected to the stimulator 'A' of the Medelec and the recording electrodes were connected to the preamplifier of the Medelec machine. The cathode and anode ports on the stimulator 'A' were reversed to ensure antidromic flow of current and reduced anodal block when the nerve was stimulated.

An optimal display of the F wave response required an amplifier gain of 250μV/div and an oscilloscope sweep speed of 5 ms/div. These were set on the Medelec machine the software in 'F wave' mode. When the 'Acquire' button was depressed, 15 stimuli were applied to the nerve at a frequency of 1Hz. This was to ensure, at least, some F wave response. The F wave response was seen on the display screen as a small CMAP (1-5% amplitude of the M wave) situated 10-20ms away from the M wave.

The mean minimum latency was displayed after each 15 recordings and noted. An average for the three sets of measured latencies was therefore recorded as this was the reading recorded on the spreadsheet.

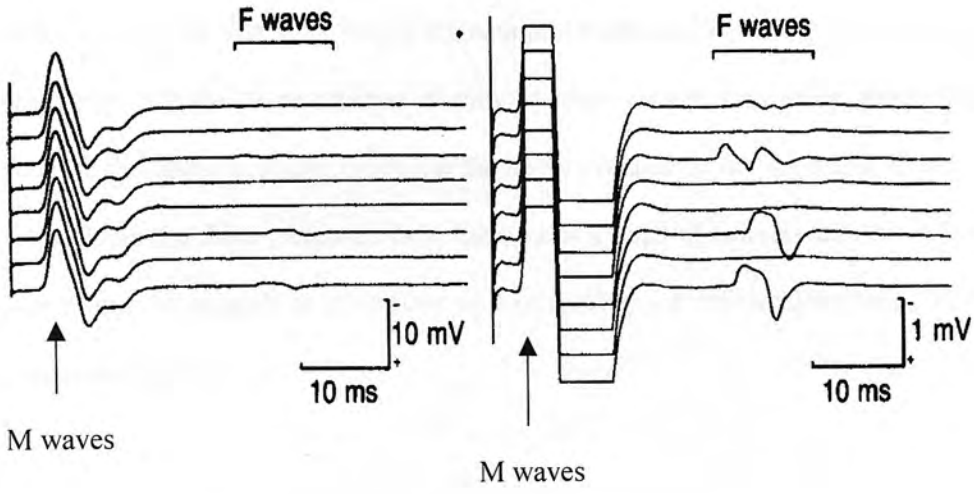


Fig 9 Figure to show the F wave response as seen on the Medelec monitor screen. The right picture shows the same F wave responses as the left picture but with the gain on the y-axis increased to 1 mV per division.

Aims of the F wave study

The F wave is due to antidromic activation of motor neurons. The longer the latency, the more distal the site of stimulation. Collateral sprouting may cause the antidromic impulse to be returned distally to cause muscle contraction prematurely because the impulse would return via the second branch of the collateral sprout. F waves were therefore measured from the control groups of animals and the end-to-side groups of animals as a possible way of looking for collateral sprouting in the regenerated nerve.

Muscle physiology

Introduction

Motor testing can be used to determine whether specific muscles or muscle groups are capable of contracting and to assess the quality of the contraction. Muscle contraction depends on the integrity of the muscle, normal passive motion in the joints across which the muscle acts, and the quality of innervation of the muscle. For a peripheral nerve surgeon the quality of innervation is extremely important. After trauma, the results of motor testing can define the extent of the primary nerve injury (Gelberman 1991b).

The structure of skeletal muscle

A single motoneurone together with the motor axon derived from it and the many muscle fibres innervated by it is known as the motor unit. The motor unit is the elementary unit of neuromuscular function. Within the muscle, the motor axon tapers

and then branches to innervate muscle fibres scattered throughout the muscle. Myelination of the nerve axon continues until it reaches the motor end-plate. It is not likely that one motor unit innervates a block of muscle fibres next to each other, but the innervation of muscle fibres is thought not to be random. It is not fully understood how the arrangement of innervation is organized however (Willison 1980).

Motor units act like biological amplifiers as one unit can innervate 10 to 1000 muscle fibres from branches of the motoneurone. A few motoneurones than can generate large isometric forces. These fibres are usually of the same type. Mammalian motor units contract at different speeds and can also be classified. The different types are: fast-twitch fatiguable, fast-twich fatiguable resistant, fast-twich intermediate and slow-twitch (Burke 1981).

Muscle fibres can be classified depending on their structural and histochemical properties. In most muscles distinct types of fibre can be distinguished, 'slow twitch' or Type I fibres rely on oxidative mechanisms for the generation of energy but a low phosphorylase and ATP-ase activity and contain low amounts of glycogen. They have a small motor unit size and a long duration of twitch action compared to Type II fibres. Type I fibres are also better at resisting fatigue compared to Type II fibres.

'Fast twitch' or Type II have mostly opposite properties with a high concentration of glycogen and (Dubowitz & Pearse 1960). Type II fibres can roughly be subdivided into Types IIa and IIb but in reality there is more of a spectrum between the two extremes. Type IIa fibres rely on oxidative mechanisms for energy generation whereas Type IIB fibres rely more on glycolysis for energy generation. Type IIa fibres are more fatigue resistant than Type IIb and small muscle fibre diameters compared to type IIb. A third type termed 'intermediate' was proposed by Close in 1972 (Close

1972). These types reflect differences in fibre function and human muscles are mixtures of the different fibre types. The muscle's ability to sustain a contraction is dependent on the pattern of energy metabolism. Muscle fibres with oxidative metabolism are said to be fatigue resistant as they are able to maintain substantial tension for some minutes. Those fibres with glycolytic metabolism are said to be 'fatiguable' as they fatigue rapidly in the face of sustained stimulation (Slater & Harris 1988).

Changes in the muscle after nerve damage

When a nerve supplying a muscle is cut, nerve-stimulated contractility of the muscle is lost and atrophy occurs. Within 3 weeks of denervation the neuromuscular junction is destroyed (Jaweed, Herbison, & Ditunno 1975). Gross features, which distinguish denervated from normally innervated muscle, are a change in colour, and a reduction in size and weight. Experiments performed on the Australian opossum by Sunderland and Ray in 1950 showed that there was rapid initial loss of weight of the muscle after denervation in the first 30 days amounting to 30%. This increased to 50-60% by day 60. The process was thought to slow significantly during the third and sixth months, a stable state being reached by 4 months and the weight loss varying between 60-80% from then onwards (Sunderland & Ray 1950). There seems to be no constant relationship between the degree of atrophy and the duration of denervation (Jennekens 1982). The rate at which atrophy occurs varies between different species, between different muscles within the same animal and different fibre types within the same muscle. It is generally accepted that Type II fibres atrophy earlier and more rapidly than Type I (Karpati & Engel 1968; Niederle & Mayr 1978), but some other

authors disagree (Jaweed, Herbison, & Ditunno 1975). Histologically there is considerable reduction in the amount of contractile substances in the fibres but they remain defined and well differentiated from the surrounding tissue. Denervation induces a relative increase in the connective tissue content of the muscle in the form of thickening of the peri- and endomysia. This thickening becomes obvious after 2 months. Even after 485 days' denervation (opossum) there was no significant reduction in muscle fibre numbers and Gutmann (Gutmann & Young 1944) did not observe degenerative changes in human muscle until 3 years after denervation. Eventually, degenerative changes occur with progressive thinning and obliteration of the muscle fibre, nuclear clumping, swelling, vacuolation and disintegration of the fibre. The final stage is reached when all trace of organized muscle fibres is lost; the original structure now being replaced by strands of fibrous tissue and columns of fat cells (Sunderland 1978a).

The physiological changes in skeletal muscle characteristic of denervation are:

1) Flaccidity and paralysis
2) Loss of the capacity to prevent hyperneurotization by foreign nerves. Nerves implanted into normally innervated muscles are unable to form functional connections with the muscle fibres. This is because the motor axons that originally innervated the muscle render its fibres refractory to the formation of new additional end plates. Denervation removes this property, as implantation of nerves into denervated muscle is followed by the formation of new functional connections which may be sited anywhere along the denervated fibre.
3) Immediately after denervation of the muscle, the resting membrane potential rises from about –75mV to about –60mV, approaching the threshold for action potential

generation and is one of the factors which accounts for spontaneous fibrillation of denervated muscle. This persists for 14 days when it falls to values below normal, the reduced threshold then persisting throughout denervation. Fibrillation is the spontaneous activity that develops in denervated muscle and persists as long as contractile elements survive. The onset of fibrillation depends on the species, size and weight of the animal, muscle specificity, state of the muscle and level of nerve section. In man, the onset of fibrillation is 19 days in limb muscles (Sunderland 1978).

4) The membrane of the denervated muscle fibre develops a high sensitivity to acetylcholine as receptors appear in regions of the denervated muscle fibres not associated with the neuromuscular junction. It may be that these represent sites of reinnervation (Frank et al. 1975).

Reinnervation of denervated muscle

Studies in the 1940s that looked at the reinnervation of rabbit skeletal muscle after varying periods of atrophy showed that the effectiveness of the reinnervation was lessened as the period of denervation increased (Gutmann & Young 1944). This has been born out by clinical experience over the years. Modern surgical teaching states that beyond 12 months of denervation of a muscle, reinnervation is not possible (Totosy de Zepetnek et al. 1992). However, the relationship between the time-course of denervation and recovery of function is not well established.

Despite denervation of several years' duration, muscle tissue will survive in a grossly atrophied but histologically recognizable form (Gutmann 1948). It is beneficial therefore, for the limb to be kept warm, immobilized in a position of rest,

with regular active and passive exercises designed to reduce vascular and lymphatic stasis. This promotes flow of blood and lymph through the muscle (Sunderland 1978). In addition, research on the trophic and neuroprotective effects of various neurotrophic factors, such as NGF, LIF, CTNF and BDNF has shown some linkage with peripheral nerve regeneration and survival of sensory neurons in dorsal root ganglia (Terenghi 1999; Yamamori et al. 1989). Another strategy has been to use sensory nerves to temporarily innervate motor targets. A study using rats in 2001 showed that the wet weights of muscles were significantly higher when sensory protection was employed compared to when it was not, but that the axon counts were not significantly higher in this group compared to the non-sensory protection group (Papakonstantinou, Kamin, & Terzis 2002).

It is still not entirely clear for what period of time denervated muscle retains its capacity to recover its physiological properties and useful function on reinnervation (Kobayashi et al. 1997). However, it is known that the recovery of function of a target muscle that follows nerve repair may be inversely related to the denervation time (Fu & Gordon 1995). After prolonged denervation, the contractile response of the muscle fibres to electrical impulses transported by the regenerating nerve axons diminishes (Finkelstein, Dooley, & Luff 1993).

Poor regeneration after prolonged denervation is not due to inability of the long-term denervated muscle to accept reinnervation because regenerated axons can reinnervate three- to five more muscle fibres than normal (Fu & Gordon 1995). Neuromuscular transmission is usually restored within 2 days after the arrival of the regenerating axon. Regenerating motor axons often reinnervate denervated muscle fibres at the site of the original neuromuscular junction. This tendency appears to be

related to the presence of an accumulation of N-CAM and of factors in the basal lamina that induce the growing axon tip to differentiate into a presynaptic terminal (Sanes, Marshall, & McMahan 1978).

Reinnervation is very effective in terms of recovery of muscle function, if it occurs soon after the nerve has been injured. This becomes less as the time between injury and the arrival of the regenerating axons at the muscle increases (Seddon 1975). According to Fu and Gordon's experiments in 1995, the primary cause of the poor recovery after long-tern denervation was a profound reduction in the number of axons that successfully regenerated through the deteriorating intramuscular nerve sheaths. Muscle force therefore progressively declined and is further compromised by the incomplete recovery of muscle fibres from denervation atrophy (Fu & Gordon 1995).

The size and distribution of motor units is known to change after denervation and reinnervation. It has been shown that denervation and subsequent self-reinnervation of the soleus muscle in cats causes a change in the distribution of values of motor unit tension. After reinnervation the motor unit tension values tended to be higher (Bagust & Lewis 1974). There is a tendency for axons to reinnervate muscle fibres that are more closely situated to each other. Regenerating axonal sprouts are more likely to make contact and innervate adjacent denervated fibres than distant ones. Packing density of fibres increases indicating that motor unit territories reduce (Kugelberg, Edstrom, & Abbruzzese 1970). Similar fibre types therefore tend to be grouped together after reinnervation. This is because different types of motoneurone tend to innervate a single type of muscle fibre (Close 1972).

Muscle contraction

The basic contractile unit of a striated muscle cell is the sarcomere, which consists of a centrally located array of thick filaments of a protein called myosin, that interdigitate with thin filaments. These thin filaments consist of polymers of actin, tropomyosin and nebulin, plus a calcium binding regulatory protein troponin and are attached to the cytoskeleton at each end of the sarcomere. Muscle fibres contain many sarocmeres in series, and muscle cells contain large numbers of muscle fibres in parallel. The movement of the thick filaments and the thin filaments over each so they become overlapped constitutes contraction.

Force generation depends on the length of the muscle. A stress-versus-length curve can be plotted by fixing the length of the muscle and measuring the maximal force during contraction. Such a contraction at constant length is termed 'isometric'. Force varies with the size of the muscle, and it is best expressed as a stress (force/cross sectional area of the muscle) to allow comparisons among muscles. Correlating the structural and mechanical relationship in muscle shows that active stress is proportional to the overlap between thick and thin filaments in a sarcomere. As the muscle is stretched beyond the length (L_0) at which maximal active force (F_0) is developed, active stress decreases linearly with decrease in overlap between thick and thin filaments. Force declines at sarcomere lengths less than L_0 as well. The optimum initial length of a muscle for maximal twitch tension is close to the resting length and for producing maximal tetanic tension a length of 5-10% greater than for twitch tensions is optimal (Rack & Westbury 1969). Another basic relationship that characterizes the output of a muscle can be obtained when the load or stress on a muscle is held constant and the shortening velocity is measured. A contraction at

constant load is termed 'isotonic'. Shortening velocity is measured by the load on the muscle. Muscles lift a heavy load slowly, shorten rapidly when lightly loaded, and exhibit their maximal shortening velocity (V_0) with no load.

Isometric muscle contraction

The maximum isometric tetanic tension at optimal length is a measure of the intrinsic strength of the muscle and is often expressed as the total force developed or the tension per gram of muscle, but for comparative purposes it is necessary to express the tension in terms of the amount of contractile tissue operating in parallel within the muscle. It is therefore commonly measured as the tension per unit cross-sectional area of muscle (Close 1972).

Isometric contractions are usually used in experiments on muscle strength because it is easier to set up a situation where the length of the muscle can be fixed between two points and the tension produced in that muscle measured, than it is to produce a situation where the muscle is working against a constant load (isotonic contraction). The recordings from isotonic contractions may not be as useful as isometric contractions when assessing muscle strength as in the former work is done and energy is used in overcoming frictional forces and/or overcoming the inertial load of adjacent inactive fibres. The total tension developed in a muscle as a result of an isometric twitch contraction is the sum of passive and active tension, where the passive tension is the tension in unstimulated muscle. Passive tension increases as the muscle is stretched. Active tension is that produced during active contraction of the muscle.

The maximum isometric tetanic tension of fast and slow muscle decreases with a decrease in temperature for contractions elicited by either indirect or direct

stimulation. The maximum tension decreases by about 30% with change in temperature from 35-37 °C to 20 °C in cat, rat and mouse muscles, and the change in tension is linear in some muscles and nonlinear in others. Fast and slow muscles differ in the temperature dependence of peak twitch tension. When the temperature is decreased from 35-37 °C to 20 °C the peak twitch tension is almost doubled in fast muscles where it undergoes little or no change in slow muscles (Close 1972).

The tension produced during an isometric contraction is dependent on the number of active motor units and also on the frequency of contraction of those motor units. Different types of motor unit contribute differently to the total tension produced by a muscle. For example, the slow motor units of a cat medial gastrocnemius make up 25% of the total motor unit population of that muscle but contribute only 5% to the total isometric force.

The mechanical response to a single action potential is a twitch. Sufficient calcium ions are released by the potential to activate all thin filaments of the sarcomere. The force of a twitch is much less than the maximum that can be developed because the release of calcium is too brief to allow generation of maximal force (Ganong 1991a). A tetanic contraction in a muscle is produced through repetitive maximal direct or indirect stimulation at sufficiently high frequency to produce a smooth summation of successive maximum twitches. Their tensions combine to produce a steady, smooth maximum contraction of the whole muscle. This can also apply to maximum voluntary muscle contractions providing their frequency is such that successive twitches of individual motor units fuse smoothly. Force is increased by the recruitment of more motor units and the maximum force that can therefore be generated by a skeletal muscle is a contraction, which recruits

all motor units to tetanize (Ganong 1991b). The total tension developed during an isometric twitch contraction is therefore much smaller than the tension produced during an isometric tetanic contraction. The development of tension in a muscle is also due do the passive series-elastic component of muscle, which is found partly in the tendons and partly distributed along the muscle fibres. If the tension during a twitch were developed instantaneously, the force recorded at the tendon would rise slowly because of stretching of the elastic component. The duration of the twitch contraction is such that the developing tension never reaches values anywhere near those of tetanic contractions.

Method of measuring twitch and tetanic muscle contractions

After completion of the electrophysiological elements of assessment of nerve regeneration as, the contractile ability of the muscle innervated by the normal or regenerated nerve was assessed.

For the normal groups of sheep where normal twitch and tetanic tensions for the ulnar and median nerves were being recorded to compare against the experimental groups (all other groups), the flexor carpi ulnaris (FCU) and the flexor carpi radialis (FCR) muscles respectively were used. For the end-to-side and double end-to-side groups both muscles were tested, as it was important to test for damage to the donor ulnar nerve during end-to-side neurorrhaphy. For the other experimental groups the FCR muscles was tested to compare their strength to the end-to-side reinnervated muscles. These other experimental groups were the ones using commonly accepted methods of nerve repair.

Electrical apparatus

The electronic equipment that was needed in order to assess the contractile properties of the FCR muscle included the Medelec machine (signal generator), a stimulus isolator, a force transducer and an oscilloscope.

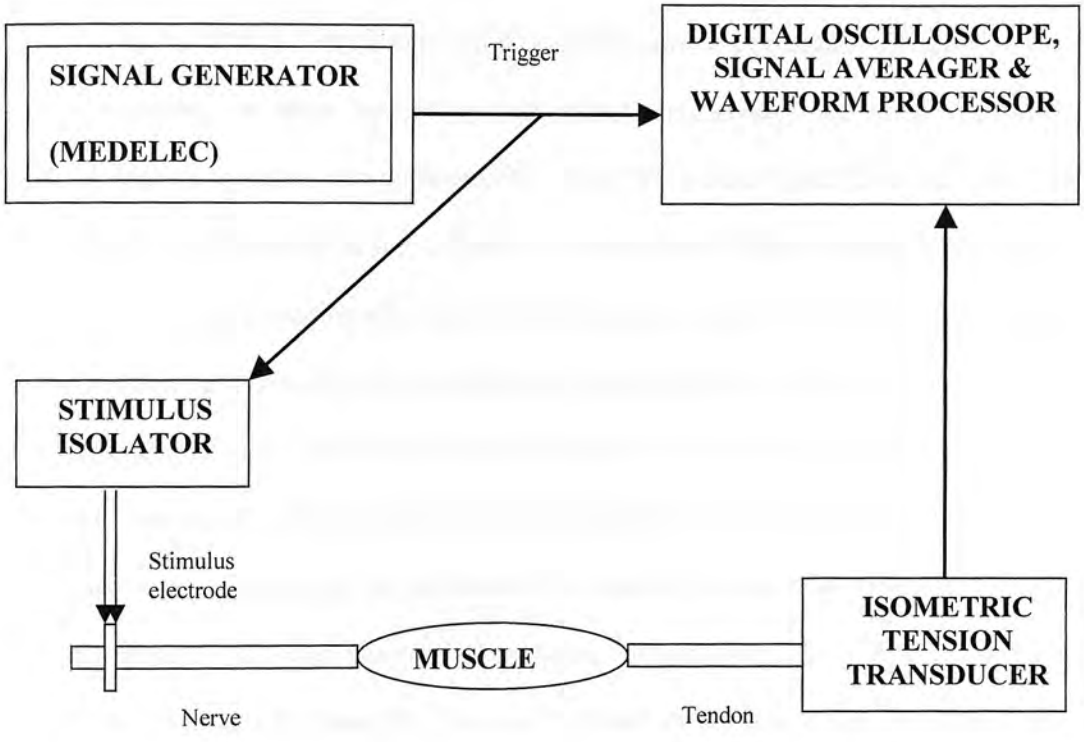


Fig 9 A circuit diagram to show the set up of the electronic equipment used for recording twitch and tetanic tensions of the muscles.

A diagram of the experimental set-up is shown in the figure above. The Medelec machine was used to generate analogue square wave transistor/transistor logic (TTL) output signals. These square signals were delivered to an optically coupled battery powered stimulus isolator unit (Digitimer, Stimulator model DS7, England), which set the width of each pulse of current to 100μs and allowed the amplitude to be varied between 1 and 100mA. For our experiments the amplitude was set to 100mA. Stimulators can be constant current or constant voltage. In our experiments we used constant current stimulators, as they are better for serial stimulation to assess nerve excitability. The TTL output signal from the stimulus isolator was delivered to the Y-input of a multichannel digital storage oscilloscope with a signal averaging and waveform-processing capability (Gould 7074, Gould electronics Ltd, Ilford, U.K.) synchronized with the delivery of the stimulus. The set electrical stimulus from the stimulus isolator was delivered to the nerve via a platinum bipolar plastic-encased electrode (similar to that used previously). The muscle was attached by its tendon to the recording beam of an isometric tension transducer (#52-9503, Harvard instruments, Edenbridge, U.K.). The subsequent twitch of the muscle caused a force on the beam of the transducer, this signal was sent to an amplifier, which was then fed into the y-input of the oscilloscope. An amplifier acts to gain or amplify an input signal before subsequent output. A good amplifier has high input impedance, where impedance is resistance to AC current.

A display of the twitch tension on a channel of the oscilloscope was recorded on its screen. The waveform processing facility of the oscilloscope allowed several measurements to be made from the displayed trace. The waveform processor of the oscilloscope is essentially software that is programmed to record certain

measurements. A remote control connected to the oscilloscope display allowed a cursor on the screen to be moved along the trace for measurements to be read off the screen. It also allowed calculation of the area under the curves of the twitch and tetanic traces.

Calibration of the tension transducer

In order to convert the values of voltage output from the isometric tension transducer, which were recorded on the oscilloscope, to units of force (newtons- N) a calibration graph was plotted. The transducer was mounted so that its beam was parallel to the floor. Small weights in the range 5-55 g were suspended from the beam. The DC output of the tension transducer was connected to channel on the oscilloscope. The deflection on the oscilloscope (V) was measured for each weight at 10 g intervals and a graph plotted of mass x acceleration due to gravity (i.e. weight / N) against oscilloscope deflection / V, using statistical software. The best-fit straight line was drawn through the points and using statistical software the equation of the line was calculated (Statistica, Stat soft, version 6). The calibration curve is shown on the next page.

Equation of the line: $y = 2.593x + 0.037$

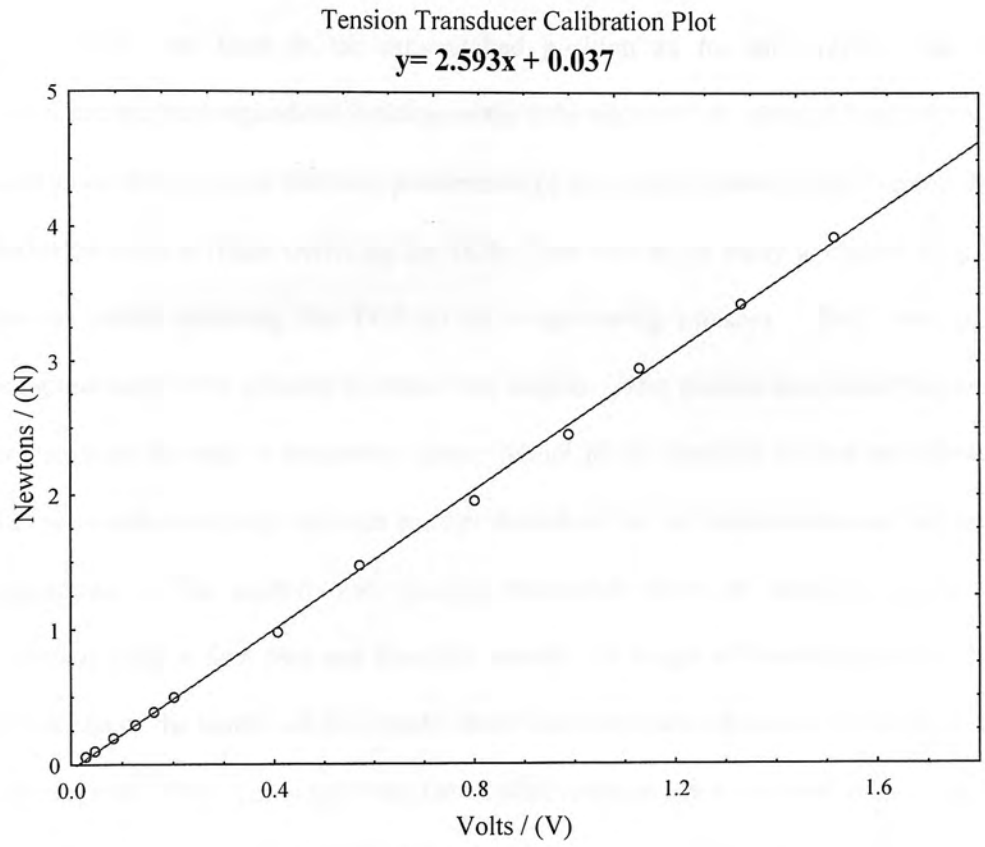


Fig 10 Graph to show the calibration curve for the force transducer with force (N) plotted against oscilloscope deflection (V).

Exposure of the FCR muscle

With the limb in the outstretched position as for the previous set of experiments, the longitudinal incision on the volar aspect of the sheep's forelimb was continued distally up to the bony prominence of the sheep's elbow. This revealed the loose connective tissue overlying the FCR. This was swept away to expose a tight fascial plane enclosing the FCR to its neighbouring muscles. This was split longitudinally with scissors to reveal the muscle. The tendon was identified and traced back through a connective tissue tunnel to its insertion around the elbow. Large vessels overlying this area were protected so that the muscles did not become ischaemic. The tendon was sharply transected from its insertion to leave approximately a 5cm free end from the muscle. A length of linen was tied to the distal end of the tendon of the muscle about 1cm from the end leaving two long ends of the linen. This 1cm length was then folded over and tied on itself with another length of linen. This ensured that the tendon was securely tied to a length of inextensible material.

Mechanical apparatus

The limb was released from the gutter rest to which it had been attached and allowed to hang freely. The forelimb hoof was pre-drilled with a 4.5mm power drill. A Steinmann pin was inserted at 90° through the keratinous parts of the sheep's cloven hoof leaving an equal part of the pin each side.

When assessing the twitch and tetanic tensions it was necessary to have the limb stretched out in such a way that contraction of all the muscles supplied by the nerve did not produce any movement of the limb. It was found that such 'rebound'

movement of the limb caused artefact on the twitch and tetanic tension traces produced on the oscilloscope.

To correct this the following set-up was devised: A G-clamp was securely fastened to the edge of the nearby workbench that was securely attached to the wall. The G clamp had a hook device soldered on to it previously. A length of inextensible plastic-coated steel cable with a loop made at either end was attached to two karabiners, one at each end. The steel cable had a cable tensioner at one end. One karabiner was attached to the hook on the G-clamp and the other was attached to the Steinmann pin between the toes of the sheep's hoof. The operating table was then moved away from the workbench to which the G-clamp and steel cable were attached to put traction on the limb of the sheep so that it was taut. Traction was adjusted by means of the tensioner according to how much artefact showed on the twitch tension trace.

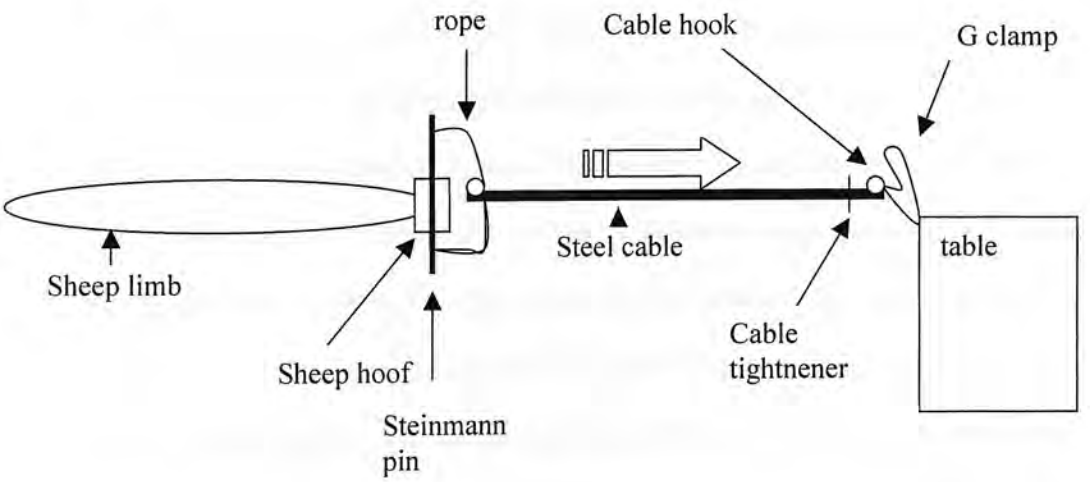


Fig 11 Diagrammatic representation of the apparatus used to measure twitch and tatanic muscle tensions.

The two ends of the length of linen tied to the muscle were knotted about 6cm from the end attached to the muscle to form a loop the end of which was placed over the part of the tension transducer receiving the force of the muscle contraction (the beam) (#52-9503, Harvard instruments, Edenbridge, U.K.). This recording beam was in the form of a metal prong protruding from the main part of the recording box. The transducer was mounted on a heavy photographic studio stand that allowed firm fixation to the floor so that no movement could occur in it during muscle contraction. The beam of the transducer was parallel with the floor. The extensible arm of the photographic stand on which the transducer was mounted could be adjusted to put tension on the muscle.

It was necessary to have the same amount of tension on the muscle as that seen when it lies normally in the sheep's body. It was therefore important to standardize the length of the muscle because the force of contraction of a skeletal muscle varies as the initial length. This is to do with optimal positioning of the contractile filaments of the muscle, which is not achieved if the muscle is stretched too far. A muscle contracts optimally when all the cross-bridges of the actin and myosin filaments are aligned and this has been shown in experiments to occur when the length is at 1.5 times its resting length. To standardize the length and therefore tension of the muscle for each twitch the muscle length including its tendon to the point where the tendon was cut was measured with a ruler before the tendon was cut and mounted. The length of the piece of tendon folded over to attach to the length of linen was noted and the muscle was mounted on the transducer beam. Using the extensible arm of the stand the muscle pulled out to the appropriate length for optimal contraction.

The transducer was connected to the y-input of the oscilloscope. The sweep speed was set at 50ms f.s.d. and the y-axis representing the muscle contraction in volts was set at 0.5 or 1 V per division depending on the size of the contraction of the muscle. The stimulating electrode that had been distal to the neurrorrhaphy sites in the experimental groups was left *in situ* and the proximal electrode was removed. This was to make sure that no current would be blocked thorough the repair sites and the muscle contracted at its maximum potential. Experience showed that a stimulating current of 100mA was always supramaximal.

The Medelec machine was set onto external stimulator mode in the 'twitch tension' part of the test menu of the software and a single stimulus was delivered to the median nerve. This caused a single twitch contraction of the FCR. From preliminary experiments it was found that the isometric tensions could vary considerably, therefore, using the signal averaging facility of the waveform processor, an average of eight traces was recorded.

It was noted at the time of muscle contraction whether there was any excess movement of the sheep's limb and whether this caused the recorded twitch trace on the oscilloscope to contain artefactual undulations from its baseline. If this was the case the traction on the limb was increased. The muscle stimulation was repeated again but care taken not to repeat this too many times so as to cause muscle fatigue and sub optimal force generation from the contraction. Below is a picture of a typical twitch contraction as it was shown on the oscilloscope screen.

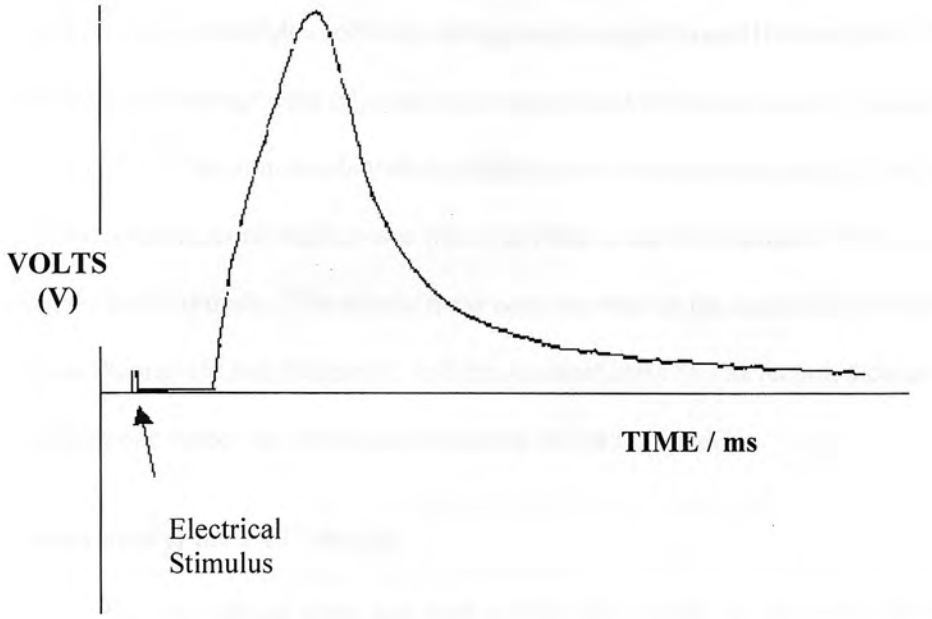


Fig 12 Graphical representation of a typical compound muscle action potential (CMAP) from FCR. This twitch contraction trace was recorded on the oscilloscope. The interval between the electrical stimulus and the beginning of muscle contraction is called the 'latent period'.

To assess the tetanic tension of the muscle the tension on the muscle was checked and the Medelec software setting was changed from 1Hz to 100Hz. The oscilloscope settings were adjusted to a sweep speed of 20s and on the y-axis to 2 V per division. The stimulus button the Medelec was then pressed and this delivered a repetitive stimulus of 50μs square waves at 70Hz to the FCR muscle, which set up tetanus in the muscle. The tetanic trace was recorded on the oscilloscope screen. When the muscle had fatigued to half its maximal force (V) as recorded on the oscilloscope screen the stimulus was turned off on the Medelec.

Assessment of the FCU muscle

For the normal ulnar and both end-to-side groups of sheep the twitch and tetanic tensions of the FCU muscle were measured. If there had been permanent axonal damage during end-to-side neurorraphy, finding an abnormal force of muscle contraction may be an indication that this had occurred.

It was desirable to assess the strength of FCU in the all the groups mentioned above but in the end-to-side experimental groups the ulnar nerve had to be transected in order to achieve a satisfactory CMAP from the ulnar to median end-to-side pathway that had been created and from which a maximum conduction velocity could be measured. If the ulnar nerve was not sacrificed the CMAP recorded from the ulnar to median pathway on the Medelec screen was a combination of the FCR and FCU muscles contracting together. Therefore the ulnar nerve was transected distal to the end-to-side neurrorrhaphy site. In nearly half of the single end-to-side experiments the FCR muscle was severely atrophied and no contraction could be seen in it even when it was stimulated directly with a bipolar hand-held stimulator. In these cases the FCU muscle twitch and tetanic tensions were measured.

To gain access to the FCU muscle the same incision was made as that used to gain access to the FCR muscle, a continuation of the longitudinal incision in the forearm distally. For the end-to-side groups the same incision was used to access the FCU muscle complex as was used to gain access to the FCR muscle. Again loose connective tissue was dissected away to reveal a tough fascial layer covering the FCU muscle. This was longitudinally split and the FCU muscle identified and carefully dissected way from the surrounding musculature, which is also supplied by the ulnar nerve. This was known from observation of muscle contraction on stimulation if the ulnar nerve in normal sheep. In most animals it was seen that the FCU muscle was bipennate. The two flat tendons associated with the muscle were sharply dissected from the insertions near the elbow area of the sheep. Because these tendons were a lot flatter and bigger than that of the FCR, a 1/0 suture was passed through the tendons together. Approximately 5 or 6 throws were used to secure the tendons and two long ends of the suture were left with the needle removed. The two ends were tied together to form a loop and this loop was placed over the beam of the transducer as for the FCR muscle. Twitch tension and tetanic tension were carried out as for the FCR muscle.

Measurements recorded on the isometric myogram

Using the wave processor of the oscilloscope, various measurements were made from the averaged trace, using the display of the timing pulse as a reference trace for the stimulus.

The measurements taken were as follows:

The maximum isometric twitch tension (F) is the maximal tension developed in a muscle as a result of stimulation by applying a single supramaximal stimulus

expressed in newtons. Maximum isometric twitch tension is a measure of the maximal force that can be produced by a muscle and according to Hartree and Hill (Hartree & Hill 1921), is the most fundamental expression of the mechanical response. Force produced by a muscle is directly proportional to its cross-sectional area and many authors express the tension produced by a muscle per unit cross-sectional area. However, it is expressed in these experiments in terms of the whole muscle, in newtons. This gives an indication of the atrophy of the muscle, but not necessarily loss of muscle fibres.

The maximum tension produced in the muscle is also an indicator of the heat produced in the muscle per unit length for short stimuli. This is based on the fact that heat is energy and is produced as a result of doing work. Work is the product of force and distance. The ratio of heat production to peak tension for an isometric twitch is constant for all temperatures.

Time to peak tension (*t*). The includes the conduction time along the nerve, the time for neuromuscular transmission and the time for the passage of the action potential to spread via the muscle membranes and cause maximal contraction.

Time to half relaxation (*r1/2*). This is the time from the start of the stimulus to the time on the downslope at which the tension of the muscle had relaxed to half of its maximal value. The end-point of contraction is sometimes difficult to define, particularly after nerve repair when the twitch is drawn out (Hems & Glasby 1993). It therefore gives a more accurate way to compare the muscles form the different experiments by measuring the time for the muscle to relax to 50%.

Time tension index (TTI). The time tension integral (TT) is the area under the curve to half muscle relaxation and was calculated using the waveform processor facility associated with the oscilloscope. This number was divided by the duration to half relaxation of the twitch to give the TTI. This value is an average of the tension produced in the muscle up until the time when it has relaxed to half its maximum value. The time tension integral has been used in previous studies as an indicator of the heat produced in the muscle and thus work done during contraction of the muscle and the ATP consumed (Fullarton et al. 2002; Hems 1993). The ATP consumed during muscle contraction, is proportional to the number of cross-bridges turning over in the muscle fibre (Hartree & Hill 1921). ATP consumption (energy and thus heat) is proportional to the turnover of crossbridges (Work = force x distance). However Hartree and Hill found that the integral was not proportional to heat production for short stimuli (Hartree & Hill 1921). This is likely to be due to the fact that the rate of heat production is not constant during a muscle twitch. A closer relationship between heat production and mechanical response of the muscle is that between heat production and maximum isometric tension. The heat produced in a muscle for a set time interval is proportional to tension multiplied by the time of contraction when the muscle is in a steady state of contraction.

The time tension integral, however, still represents an important mechanical property of the muscle. It is proportional to the momentum that would be produced if the muscle were attached to a large mass (m) hanging at rest from a vertical string. This information gives an idea of the ability of the muscle to move heavy masses.

Peak tetanic tension (N). The twitch tension value is probably not as useful an index of recovery of muscle function as the value of tetanic tension. This is because the maximum tension that can be produced by a muscle is not necessarily produced in a twitch contraction. Therefore, the values of the twitch tension are much more variable with changes in the stimulus and the environment. The twitch/tetanic ratio of extensor digitorum longus muscle in adult rats was reported as 0.19 (Close 1972).

Aims of the twitch tension and tetanic tension studies

The force generated in an isometric twitch provides information about the amount of ATP that muscle needed to contract. Therefore, the force generated in a muscle that is denervated and then re-innervated, after surpamaximal stimulation gives an idea of how many muscle fibres were re-innervated after nerve repair. This indirectly provides information on the quality of the nerve repair, as axons need to negotiate repair sites in order for nerve regeneration to advance toward the end-organ.

Wet muscle weights

The morphological changes seen in the muscle fibres, after denervation and reinnervation, have an impact on the level of recovery of function. Muscle weakness may be a consequence of muscle fibre atrophy and/or the proliferation of connective tissue. The inability of muscles to contract rapidly or to maintain prolonged activity may be a consequence of a change in the relative abundance of fibre types present. Comparison of the wet weights of the FCR muscles between each group and the FCU muscles in the end-to-side groups compared to their normal values, gives us an indication of the degree of atrophy or loss of fibres after denervation and reinnervation.

Loss of weight is a progressive process after denervation and is permanent if the muscle is not re-innervated (Sunderland & Ray 1950). The weights may also, then, give us an idea of how successful regeneration in the nerve has been.

Removal of tissue

The FCR and FCU muscles were removed from their proximal attachments using a scalpel. Care was taken to ensure that the muscles were removed as a whole piece leaving none of the muscle behind. The FCR muscle was relatively easy to remove as a whole piece owing to its being a single structure with a single proximal bony attachment. The FCU muscle was more difficult being bipennate but its parts were carefully identified each time so that an equal amount of muscle was removed each time. After the tendon for each muscle had been carefully identified and then cut, the muscle was held taut to allow easy dissection of the muscle away from its surrounding connective tissue and proximal attachment. The muscles were weighed on an electronic balance, which had been zeroed before placement of the specimen on the tray of the balance. The weights were recorded in grams (*g*). For the normal ulnar and normal median groups of animals, the FCU from the ulnar group and the FCR from the median group were removed. For both end-to-side groups both muscles were taken and for all the other experimental groups the FCR muscles only were taken.

Histological methods

Removal of tissue

For each group of animals, nerve tissue was removed for histological assessment.

For the normal groups of animals, a 2cm length of normal nerve was removed for the single end-to-side group a 1cm section of nerve tissue was taken from the ulnar nerve proximal to the neurorraphy site and a similar 1cm section was taken from both the ulnar and median nerves distal to the neurorraphy site. For the double end to side group, 1cm sections were taken from ulnar and median nerve proximal to the neurorraphy sites and again a similar 1cm section was taken from median and ulnar nerves distal to the neurorraphy sites. For all the other experimental groups 1cm sections were taken proximal and distal to the neurorraphy sites.

Proximal sections as well as distal sections were taken because degeneration and regeneration occurs proximal to the site of injury and repair for a short distance, as well as Wallerian degeneration occurring distally.

For the double end-to-side group a section of the tissue situated between the two neurorraphy sites was dissected out and taken for examination as well.

Fixation of tissue

Specimens of nerve tissue were processed by a standard method similar to that described by Miko and Gschmeissner in 1994 to produce resin-embedded sections (Miko & Gschmeissner 1994). Modification was required to ensure adequate impregnation with osmium and resin infiltration in the larger specimens.

Immediately after removal from the animal, the nerve specimens were placed for 45 minutes in 4% glutaraldehyde in 0.1M sodium cacodylate buffer at room temperature for initial fixation and to make the tissue firmer for cutting. The nerve was next trimmed of any excess connective tissue and damaged ends and then cut into 1mm thick transverse slices using a razor blade. The slices were replaced in glutaraldehyde at room temperature for a further 2 hours before washing with 10% sucrose in 0.1M sodium cacodylate buffer. Each specimen was placed in a few millilitres of the sucrose solution left on a rotor for 20 minutes. This washing process with sucrose occurred two more times after this.

The specimens were then placed in 1% cacodylate buffered osmium tetroxide for 3 hours. This step is essential for myelin preservation. Shorter times were found to be associated with inconsistent fixation of myelin in the middle of the nerves. Sometimes, for the slightly larger diameter specimens a further hour in the osmium was required. Further time in the osmium was also required if the specimen still appeared colourless in patches to the naked eye; it should have been completely opaque.

After the specimens were opaque (usually after three hours) they were washed in 10% alcohol. For each tube containing the specimens, the osmium solution was removed using a pipette taking care not to remove or damage any of the sections of nerve. Each specimen tube was filled three quarters full with 10% alcohol solution and placed on the rotor. This was repeated twice more at 20 minute intervals. This was to remove or 'wash' any excess osmium and to dehydrate the specimens. This washing process was repeated using 100% alcohol instead of 10%. Again, three changes of alcohol took place at 30-minute intervals with specimens placed on the rotor between

each change. The specimens were then placed in polypropylene oxide solution for 30 minutes on the rotor.

Embedding

The specimens were next placed in fresh Araldite at room temperature overnight. An ice cube tray was used to separate each specimen. Each specimen was clearly marked by marking a grid representation of the ice cube tray out on a piece of paper.

The next day each nerve section was removed from the Araldite and orientated and embedded in fresh Araldite in Araldite moulds in turn. Two slices of nerve section were placed in each section of the Araldite mould, one at each end so that its flat surface lay up against the end. This made it easier to identify the orientation of the section slice when it was cut for histological examination. Each specimen again was clearly labelled using the grid drawing as before, before placing each mould in an oven at 60^{o} to polymerize the Araldite for at least 48 hours.

Cutting

Semi-thin (1μm) sections were cut for light microscopy using an ultrathin microtome with glass knives. The glass knives were prepared on a purpose built glass cutter from pre cut lengths of glass.

A section of nerve tissue now embedded in polymerized Araldite was removed from the mould and this block was placed on a hot plate set at 40°C to soften for 1 minute. The block was cut in half to separate the two nerve sections embedded in it. One half of these halves of the block were placed on a 1cm section of wooden dowel rod so that the flat surface of the section of nerve faced upwards

and the part of the section closest to the edge of the Araldite was topmost. This was secured in place on the flat cut surface of the rod with warm sealing wax that was melted onto a spatula from a block using a Bunsen burner.

The block now secured onto the dowel was placed under the light microscope to give a magnified view of the nerve section and excess Araldite from around the nerve section was trimmed away using a razor blade.

The nerve section block was then mounted on the microtome for trimming. A glass knife was secured into place taking care to recognise and prevent damage the cutting part. Sections were then cut from the mounted nerve until sections containing nerve specimens could be seen coming away from it. The nerve section was now ready for ultrathin sectioning. A new glass knife was used for this, discarding the previous one in a sharps bin. A small amount of candle wax was melted onto the fat surface of the glass knife to form a 'boat'. Care was taken not to get any of this wax on the blade. A drop of distilled water was pipetted into this 'boat'. The section thickness was set to 1μm using a dial on the microtome and sections were cut and floated into the 'boat'. These ultrathin sections were removed with the blunted end of a mounted needle taking care not to damage them and floated on to glass slides that contained a drop of distilled water. The sections were prevented from folding and curling using chloroform solution held near the sections in a pipette. The sections were then dried on a hot plate and looked at under a light microscope to check no damage or folding had occurred. If damage had occurred more sections were cut and the process repeated until good quality sections were achieved. Complete flat transverse nerve sections were obtained for morphometric analysis.

Staining

The stain used was toluidine blue, which stains all structures to a varying degree. A solution of toluidine blue in 1% sodium tetraborate was used. A few drops were placed over the sections on the glass slide while still on the hot plate and left until steam was just seen to start rising and the edges of the toluidine stain took on a greenish sheen. The stain was then immediately washed off with cold water and the slide allowed to dry. Cover slips were not used as the mounting medium was found to lift sections from the slide.

Microscopy

Sections were viewed using a compound microscope at a variety of magnifications. General features were noted.

Morphometric Analysis

This is a method of quantifying features of the nerve sections seen on histological examination. It was carried out on the 1μm nerve sections using a computerized image analysis system (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada) connected to compound microscope.

Fibre diameter and myelin sheath measurement

After repair of a peripheral nerve, maturation of nerve fibres takes place and fibre diameter increases as smaller fibres, which fail to make peripheral connections die. The eventual size of regenerating nerve fibres is dependant to some degree on the diameter of the parent fibre but mainly on whether contact is made with an appropriate end organ. Fibres which fail to make appropriate end organ connections

may become myelinated but may fail to reach the diameter size of a normal nerve. Whereas the nerve fibre structure of the nerve trunk returns to normal after crushing, the size, number and calibre spectrum of the fibres are not fully restored after neurotmesis, nerve suture and grafting (Gutmann & Sanders 1943). Comparison of fibre maturation in the nerves distal to different repairs therefore represents a measure of the extent to which functioning connections have been made.

When a nerve is cut and degeneration occurs, removal of the axon and myelin debris occurs by phagocytosis, and the endoneurial tubes shrink. This shrinkage is progressive as the denervation time increases. The fibres are affected in proportion to their diameter. The largest fibres are reduced to tubes no larger than 2-3μm, which represents an 80 to 90% reduction in diameter (Sunderland & Bradley 1950). Reduction in calibre of the endoneurial tube is mostly due to shrinkage rather than thickening of the tube wall. During regeneration the new axon has the ability to inflate the endoneurial tube it is trying to penetrate and grow. The shrinkage during degeneration is therefore a reversible process, which is consistent with the elastic properties of the endoneurium (Sunderland 1978).

The influence of the endoneurial tube size on the end result of regeneration has been the subject of considerable experimental investigation. These studies have shown that while large central fibres could in a limited way inflate smaller peripheral tubes, very small Schwann or endoneurial tubes in the distal stump have a restrictive effect on the growth of new axons (Berry & Hinsey 1946; Simpson & Young 1945). From this it was concluded that the regenerating axons that reinnervate tubes smaller than those they originally occupied remain of reduced size, and that this results in the permanent impairment of their conducting ability. Three months after denervation,

the largest endoneurial tubes that are available for regenerating axons will rarely exceed 3μm in diameter, suggesting that nerve repair after this time will result in permanent functionally impaired nerve fibres (Sunderland & Bradley 1950). Delays of more than 6 months between nerve severance and suture result in considerable impairment of nerve fibre reconstitution and restoration of their conduction velocity and action potentials (Berry & Hinsey 1946). Clinical studies by Sunderland also around this time, indicate that the denervated endoneurial tube retains the capacity, for at least 12 months, of transmitting axons to the periphery in a manner that does not differ significantly from that observed when suture is undertaken immediately or shortly after severance, and that muscle function may be restored after reinnervation under these conditions (Sunderland & Bradley 1949; Sunderland & Bradley 1950).

Fibre diameters increase slowly after nerve suture but may not be complete even after 4 years. Another study by Hodes (Hodes, Larrabee, & German 1948) suggested that the largest motor axons failed to grow beyond about 40% of the diameter of the largest motor axons in normal nerve. Conduction velocity in their suture experiments did not improve beyond 40% of normal. Other early studies reported restoration of up to 80% of the normal conduction velocity (Berry, Grundfest, & Hinsey 1944) and Erlanger and Schoepfle in 1946 went on to conclude that the conduction velocity of regenerating nerve fibres may never return to normal (Erlanger & Schoepfle 1946).

The changes due to prolonged denervation, which do restrict recovery, are those, which either prevent regenerating axons from reaching appropriate endoneurial tubes, or make it impossible for the peripheral tissues to respond satisfactorily when they have been reinnervated.

Generally there is a linear correlation between absolute myelin thickness and axon calibre, the largest axons being surrounded by the thickest myelin sheaths. However, myelin thickness is not constant for axons of the same diameter, so it is not possible to calculate the amount of myelin that would surround an axon of a particular diameter. In regenerating nerves the myelination of smaller fibres is less than in larger fibres. Therefore myelin sheath thickness will be different in regenerating nerves than in normal nerves. A better variable to measure to assess nerve fibre maturation would be the G-ratio, which is the relationship between the axon diameter and the fibre diameter (axon diameter / fibre diameter) (Glasby, Gschmeissner, Hitchcock, & et al 1986).

Measurements recorded

Fibre diameters were measured across the minor axes of elliptical profiles to avoid bias arsing from sections cut obliquely (Mayhew, MacLaren, & Henery 1990).

It was decided to count 200 nerve fibres in each nerve section to give a representative sample of the whole nerve, as suggested by Mayhew (Mayhew, MacLaren, & Henery 1990). This does not have the advantage of absolute accuracy associated with total fibre counts but is an acceptable way of measuring distribution of axon and fibre diameters and myelin sheath thicknesses for a number of different nerve specimens. This sample was found to be large enough to eliminate random variation on different parts of the section and so produce consistent results when repeated measurements were made on the same section.

In order to identify single neurons and allow a count of the neurones in a nerve section, it was necessary to obtain an image using an x40 objective lens. An

image of smaller magnification did not differentiate between small and closely spaced neurones.

The dry slide containing the semi-thin section of nerve embedded in Araldite was placed on a microscope platform (Jenamed Variant, Carl Zeiss). No cover slip was used, resulting in a more focused image than if a cover slip was used. A beam splitter was used with the microscope, which allowed binocular vision of the section with 10 times magnifying eyepieces resulting in an effective magnification of 400 times, and a camera (Kestril 25) image to be displayed. The camera image of the slide was with out the eyepiece magnification. This camera image was input directly to a commercial image analysis computer software package (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada).

The resulting magnified image only represented a fraction of the total nerve section. The exact magnification of this image was not known as the magnification of the camera lenses was not known. The AIS system was therefore was calibrated, this is discussed later in this section. The image of the nerve section was digitized by a camera attached to a microscope, as described above, and stored on computer as a TIFF file (Tagged Image File Format). TIFF is a compressed and flexible, platform-independent format which is supported by numerous image processing applications. 4 to 8 images of the nerve section were stored in separate TIFF files at allow for 200 nerve fibre counts. The images were selected from the slides randomly by using the first two clear images seen from each slide. These images were saved on computer hard disc and loaded into an image analysis computer software package (Analytical Imaging Station (AIS) version 3.0, Imaging Research Inc, Canada). With the AIS software, neurons on the reconstructed images were marked using a mechanical

pointing device ('mouse'). The program automatically recorded the number of marks made over the image and thus 200 neurons in each nerve section could be counted. It also measured the distance between the marks to allow a line to be drawn for axon and fibre diameter measurements (Kelly 2002).

These morphological variables were measured:

Axon diameter

Fibre diameter

After images of all nerve sections had been digitized in this way they were later analysed by the image analysis program. This system required prior calibration, and the image of a 2mm graticule with 10μm divisions was similarly digitized. The computer program recorded this calibration and this was used for all measurements to be obtained.

The myelin sheath thickness was calculated by the formula:

$$MST = \frac{(f - a)}{2}$$

Where:

MST = myelin sheath thickness (μm)

f = fibre diameter (μm)

a = axon diameter (μm)

The G-ratio was calculated using the formula:

$$Gratio = \frac{a}{f}$$

Where:

f = fibre diameter (μm)

a = axon diameter (μm)

These measurements were exported from the AIS program to a spreadsheet (Excel, Microsoft, USA), for further analysis.

STATISTICAL ANALYSIS

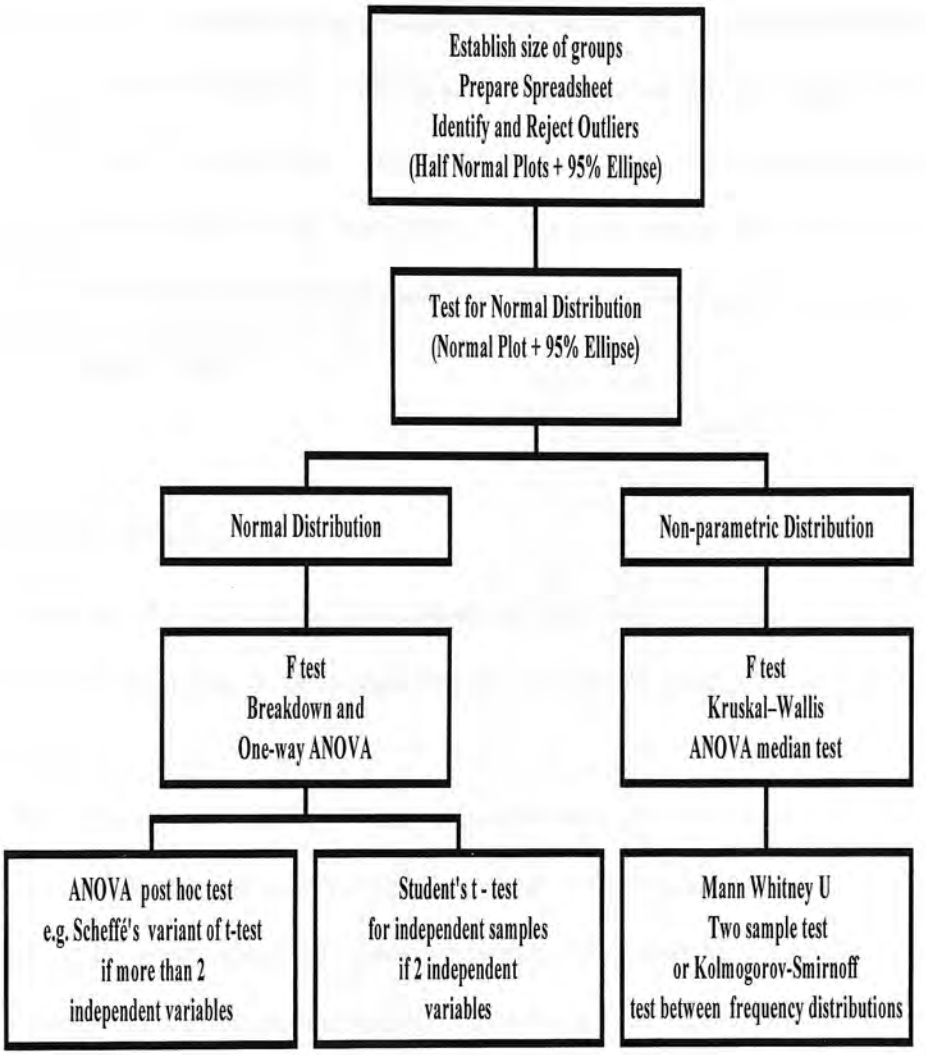
Application of statistics to raw data allows the information from experimentation to be easily understood. It allows conclusions to be drawn from data, which is distributed according to biological probability. It is therefore important to choose statistical tests that are appropriate for the experimental data and ones that will provide meaningful answers to questions posed. Statistics, however, can never prove or disprove a hypothesis. It can only give a figure that represents the probability that a result is significant.

The method of statistical analysis used in the present work is that which has been developed in the Peripheral Nerve Research Group, Department of Clinical Neurosciences, University of Edinburgh for the appraisal of models of nerve injury and their surgical repair (Fullarton 1994; Fullarton et al. 2000; Fullarton & Glasby 1997; Glasby, Fullarton, & Lawson 1997). All of the data from the present experiments considered above were treated this way.

Raw data

Raw data were first organized into a spreadsheet using the computer programme 'Microsoft Excel'. The extreme left hand column contained a list of identification numbers of the individual sheep. In a column immediately to its right the independent variable 'Experiment' was placed as a list in which the subgroups *i.e.* procedures were designated 'Normal', 'Neuro', 'Graft', 'Wrap', 'DE to S' and 'E to S' (see abbreviations page). In subsequent columns to the right of this, each dependent variable was recorded *e.g.* 'CV_{max}', 'Pk (N)' *etc.* When the Excel spreadsheet was complete with all of the morphological and physiological results it was imported into the statistics computer programme ('Statistica version 6.0' — Stat soft Inc, 2300 East 14th Street, Tulsa, O.K. 71404, U.S.A.). This programme is an extensive package for statistical analysis and graph plotting.

The statistical appreciation of how group sizes were determined has been discussed above. The figure shown below is a flowchart of the statistical analyses, which were available for the experimental data recorded in the present experiments.



A flow chart to show the steps of statistical analysis

Rejecting outliers

First, it was necessary to identify and reject outliers within the raw data. This was accomplished by first plotting 'half-normal' probability plots in which the selected variable was plotted in a scatterplot against the values 'expected from the normal distribution' for each column of data. The half-normal probability plot is constructed in

the same way as the standard normal probability plot, except that only the positive half of the normal curve is considered. Consequently, only positive normal values will be plotted on the *Y-axis*. On each plot it was possible to draw an ellipse representing the 95% confidence intervals for the distribution. Points lying outside this ellipse were considered to be outliers and were rejected from the study. The resulting spreadsheet was termed 'weeded data'.

The Normal Distribution

After rejecting the outliers the columns of data were re-plotted as normal probability plots in order to determine whether or not the data fitted a normal distribution.

The curve of a normal distribution is symmetrical about the mean and bell-shaped. Normal distributions can vary in their shape. The standard deviation shows the spread of the results about the mean and tends to be smaller for larger samples and *vice versa*. Samples with small standard deviations have a more 'peaked' bell-shaped curve than samples with a large standard deviation where the bell-shape is shorter and wider. The data obtained in the present experiments were tested for normality before any statistical tests were applied.

The way the standard normal probability plot was constructed is as follows. First, the values were rank ordered. From these ranks, Z values (i.e., standardized values of the normal distribution) were computed based on the assumption that the data came from a normal distribution. These Z values were plotted on the y-axis of the plot. If the observed values (plotted on the x-axis) are normally distributed, then all values should have fallen onto a straight line in the plot. If the values were not

normally distributed, they would deviate from the line. The fit of the computed line to the scatter of the raw data was tested by the programme using the Shapiro–Wilk *W* test. If the *W statistic,* so produced was significant, then the hypothesis that the respective distribution was normal was rejected. The *Shapiro-Wilk W test* is the preferred test of normality because of its good power properties as compared with a wide range of alternative tests (Shapiro, Wilk, & Chen, 1968). At this point, for ease of recognition, each of the columns of dependent variable data in the spreadsheet that was not normally distributed was converted to a red font for easy identification.

Statistical tests

The next two stages in statistical testing were directed at identifying the presence of differences (variants of the F test) and identifying where those differences lay (variants of Student's t test). Different algorithms had to be adopted for normally and non-parametrically distributed data. Tests on normal data are based on mean values, they are generally more sensitive than non-parametric tests and are used whenever possible. Non-parametric tests involve a process of ranking, where the true values obtained from experimentation are assigned a number depending on their position relative to other data points.

F tests

The F test is a test of variance that can be applied to all samples simultaneously. It works on the principle that if all samples are from the same population, the variation in the data attributable to the differences between the group means can be accounted for by the variation attributable to the differences between

individuals. The F test indicates whether samples are significantly different from each other, but a significant result does not indicate where these differences lie.

For normally distributed data, the F test was applied in the form of one-way ANOVA. The independent variable with its subgroups was the 'grouping' or 'factor' in the ANOVA calculation and the dependent variables were the columns of weeded data. From the ANOVA 'p' values for statistical differences were identified.

For data, which were not parametrically (normally) distributed, statistical analysis based upon the ranking of data was used. There are a variety of tests equivalent to the more sensitive tests used above. The *Kruskall-Wallis test* is a non-parametric alternative to one-way (between-groups) ANOVA. It is used to compare three or more samples, and it tests the null hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Thus, the interpretation of the *Kruskall-Wallis test* is similar to that of the parametric one-way ANOVA.

***Post hoc* tests**

In order to find where these differences lay it was necessary to perform post *hoc* tests, which compare the means of these independent groups of data. The paradigm for this is Student's t test, which compares the means of two independent samples. However in the present experiment the ANOVA disclosed multiple differences. If repeated t tests are performed on a single cohort of data, there is an increased likelihood of Type 1 error in the analysis. Type 1 error is, in effect, the acquisition of 'false positives'. In order to reduce this probability when determining differences between groups means the more conservative Scheffé test is used. This is

a variant of the t test specifically for use in the situation of analysis of variance where multiple groups are being considered.

For testing where the differences lay in non-parametrically distributed data where the Kruskall – Wallis test had been used, the Mann-Whitney U test was used. The Mann-Whitney U test assumes that the variable under consideration was measured on at least an ordinal (rank order) scale. The interpretation of the test is essentially identical to the interpretation of the result of a *t*-test for independent samples, except that the U test is computed from rank sums rather than means. The U test is the most powerful (or sensitive) nonparametric alternative to the *t*-test for independent samples; in fact, in some instances it may offer even greater power to reject the null hypothesis than the *t*-test.

The results of these tests are recorded below.

Statistical Power

In any study where statistics have been used it is important to consider the statistical power of one's tests. Statistical power is precisely defined as: 'The probability of rejecting a false statistical null hypothesis'. It is thus important in pre-determining the size of samples before undertaking experiments and, at the end, in assessing the accuracy of the analysis that was used. 'Statistica' contains a comprehensive '*Power Analysis*' module, which allows these tests to be carried out. Using this it was possible to test the power of each of the post-hoc tests where significant differences between dependent variables had emerged. It was then possible to compare these results retrospectively with the computations for sample size, which had been made before the experiments were undertaken.

CHAPTER 3 - RESULTS

ANIMALS

All animals showed progressive signs of recovery after operation. After full recovery from the effects of the anaesthetic in the recovery pens, the sheep were placed in a barn for 48 hours. During this time it was seen that all the sheep were grazing and walking without limps or any disability.

The first sheep in the end-to-side group (EDS1) had to be replaced as during the initial procedure of end-to-side neurorraphy the ulnar nerve was accidentally transected. It was replaced with another animal (EDS 13). EDS 5, the fifth sheep in the end-to-side group developed gastro-intestinal threadworms that were uncontrolled with antihelmintic therapy. The animal became progressively more unwell and it was decided the most humane course of action was for it to be humanely killed by a vetinary surgeon. This sheep was also replaced with another animal (EDS 14). EDS 9 was found dead in the open field. This animal was not replaced, as there were not the resources to do so.

The animals were observed every day in the open fields where they were kept during the time of the preliminary operative procedures and the acute procedures. A note was kept if any animal developed physical disability or failure to thrive.

There were no problems with any of the other sheep in any of the other groups during the time of the preliminary operative procedures and the acute procedures.

DISPLAY OF RESULTS

The numerical values of the results data are displayed in tables. Where possible only the tables of data representing significant results are shown. However, all results for the normal data are shown to give an idea of the size and ranges of values for each mode of nerve regeneration assessment test. Most of the electrophysiological results obtained from the Medelec are given to 2 decimal places as this is how they were displayed and the standard deviation tended to be narrow. Values from the muscle physiology were generally taken to 2 decimal places aswell but if the actual values were 3 or 4 figures with a wide standard deviation, the values were displayed to 1 or no decimal places. Axon and fibre diameters were measured in micrometres but the range of values was narrow. The values were therefore shown to 2 decimal places.

RESULTS - NORMAL GROUPS

The results of the sheep that underwent no first surgical procedure to injure and repair their median nerves are presented here and will be used as reference to compare with the results with the other experimental groups of sheep.

'Normal median' group

Electrophysiological tests

The results for the transcutaneous stimulated jitter and the maximum conduction velocity experiments performed on the normal sheep are shown here in the

table. It was found that the values obtained for each sheep were consistent with each other.

NUMBER	Expt	Date	Weight (kg)	Temp (°C)	TSJ (μs)	CV_{max} (m/s)
MCL1	NORM	280502	62	37.0	10.6	92.6
MCL2	NORM	290502	77	37.3	8.1	89.5
MCL3	NORM	50602	52	37.2	7.5	68.7
MCL4	NORM	50602	55	37.2	8.0	98.0
MCL5	NORM	60602	46	38.0	8.4	77.5
MCL6	NORM	100602	75	37.8	9.5	62.0
MCL7	NORM	100602	65	37.8	8.2	90.0
MCL8	NORM	20702	71	36.6	7.7	96.8

Table 1 Results of the electrophysiological tests performed on the normal median nerves of 8 normal sheep. TSJ denotes the jitter value in microseconds and CV_{max} denoted the maximum conduction velocity in milliseconds.

Muscle physiology

The values of the experiments performed on the muscle that was innervated by the median nerve (flexor carpi radialis) are presented in the table here.

	Expt	Date	Weight (kg)	Temp (°C)	tPk (ms)	Pk (V)	Pk (N)
MCL1	NORM	280502	62	37.0	ND	ND	ND
MCL2	NORM	290502	77	37.3	42.0	2.9	7.6
MCL3	NORM	50602	52	37.2	65.5	2.8	7.3
MCL4	NORM	50602	55	37.2	39.5	2.3	6.2
MCL5	NORM	60602	46	38.0	54.0	3.0	7.8
MCL6	NORM	100602	75	37.8	33.0	1.1	2.8
MCL7	NORM	100602	65	37.8	37.5	0.8	2.1
MCL8	NORM	20702	71	36.6	64.5	1.7	4.5

Table 2 Results of the time to peak or maximum contraction of the FCR muscle in milliseconds – Pk (ms), the peak or maximum contraction in volts – Pk (V) and in newtons – Pk (N). These readings were read from the oscilloscope trace. ND – value not determined.

Sheep Number	R/2 (ms)	ATw (mVs)	ATw (mNs)	TwTi (N)	Tet (V)	Tet (N)
MCL1	ND	ND	ND	ND	ND	ND
MCL2	92.0	189.5	491.4	5.3	7.0	18.1
MCL3	150.5	305.5	792.2	5.3	9.4	24.5
MCL4	78.5	138.0	357.8	4.6	10.0	25.9
MCL5	115.5	235.4	610.4	5.3	8.8	22.7
MCL6	77.0	51.5	133.6	1.7	9.1	23.6
MCL7	92.0	53.4	138.5	1.5	9.1	23.6
MCL8	161.0	195.6	507.2	3.1	8.4	21.8

Table 3

NUMBER	Tet/2 (s)	ATet (Vs)	ATet (Ns)	TeTi (N)	Mass (g)
MCL1	ND	ND	ND	ND	14.7
MCL2	26.0	155.6	403.5	15.5	ND
MCL3	15.4	117.6	305.0	19.8	7.5
MCL4	18.0	136.9	355.1	19.7	11.2
MCL5	20.2	165.7	429.7	21.2	8.5
MCL6	19.2	146.4	379.7	19.8	12.0
MCL7	26.6	218.6	566.9	21.3	8.3
MCL8	18.0	117.8	305.5	17.0	9.8

Table 4 Tables 3 and 4 show the results of the time to half peak amplitude of contraction and the area under this part of the twitch contraction curve as shown on the oscilloscope (R/2(ms) and ATw (mVs) respectively). Areas are measured in mVs and mNs. From this area, the time tension index can be calculated. This is an indirect measure of the work done by the muscle. A similar value was calculated for the tetanic tension achieved by the muscle. This was taken from the measurements recorded from the oscilloscope trace of the muscle in tetanus. The maximum force recorded in volts and converted to newtons (Tet (V) and Tet (N)). ND – value not determined.

'Normal ulnar' group

Electrophysiological tests

The results for the transcutaneous stimulated jitter and the maximum conduction velocity experiments performed on the normal sheep are shown here in the

table. It was found that the values obtained for each sheep were consistent with each other.

Sheep	Sheep Number	Expt	Date	Mass of sheep (kg)	Temp (°C)	TSJ (μs)	CV_{max} (ms)
OE642	UCL1	UCONT	11002	53	36.2	8.7	88.0
GL21	UCL2	UCONT	91002	55	38.8	9.7	100.0
GL24	UCL3	UCONT	161002	60	37.3	8.6	73.8
GL26	UCL4	UCONT	221002	65	37.3	7.9	ND
A1	UCL5	UCONT	111102	58	ND	6.4	102.2
A1	UCL6	UCONT	121102	58	ND	11.4	100.0
GL27	UCL7	UCONT	121102	60	37.8	9.0	68.9
GL27	UCL8	UCONT	121102	60	37.4	8.4	87.5
OE354	UCL9	UCONT	121102	48	37.8	6.8	73.7
OE354	UCL10	UCONT	121102	48	37.4	6.5	86.7

Table 5 Results of the electrophysiological tests performed on the normal ulnar nerves of 8 normal sheep. TSJ denotes the jitter value in microseconds and CV_{max} denoted the maximum conduction velocity in milliseconds. UCL and UCONT denote 'ulnar control'. ND – data not obtainable.

Muscle physiology

The results of the experiments performed on the muscle that was innervated by the median nerve (flexor carpi radialis) are presented in the table here.

Sheep Number	Expt	Date	Weight (kg)	Temp	tPk (ms)	Pk (mV)	Pk (N)
UCL1	UCONT	11002	53	36.2	41.6	1210	3.1
UCL2	UCONT	91002	55	38.8	43.0	770	2.0
UCL3	UCONT	161002	60	37.3	32.0	240	0.7
UCL4	UCONT	221002	65	37.3	31.0	1250	3.3

Table 6 Results of the time to peak or maximum contraction of the FCU muscle in milliseconds – Pk (ms), the peak or maximum contraction in Volts – Pk (V) and in newtons – Pk (N). These readings were read from the oscilloscope trace.

Sheep Number	tR/2 (ms)	ATw (mVs)	ATw (mNs)	TwTi (N)	Tet (V)	Tet (N)
UCL1	134.6	119.2	309.1	2.3	7.1	18.6
UCL2	120.0	65.3	169.4	1.4	4.6	12.0
UCL3	103.0	17.4	45.1	0.4	2.0	5.1
UCL4	69.5	54.0	140.1	2.0	5.7	14.9

Table 7

Sheep Number	Tet/2 (s)	ATet (Vs)	ATet (Ns)	TeTi (N)	Mass (g)
UCL1	12.4	67.9	176.1	14.2	35.9
UCL2	18.0	65.0	168.6	9.3	39.8
UCL3	9.2	14.0	36.3	3.9	33.2
UCL4					47.0

Table 8 Tables 7 and 8 show the results of the time to half peak amplitude of contraction and the area under this part of the twitch contraction curve as shown on the oscilloscope (tR/2(ms) and ATw (mVs) respectively). Areas are measured in mVs and mNs. From this area, the time tension index can be calculated. This is an indirect measure of the work done by the muscle. A similar value was calculated for the tetanic tension achieved by the muscle. This was taken from the measurements recorded from the oscilloscope trace of the muscle in tetanus. The maximum force recorded in volts and converted to newtons (Tet (V) and Tet (N)).

Summary of normal results

The tables below show summaries of the results of the all the tests undertaken on the normal median and normal ulnar nerves.

Descriptive Statistics (normal median nerves)

	Valid N	Mean	Minimum	Maximum	Std.Dev.
AxonM	5	8.98	8.46	9.60	0.55
FibreM	5	16.71	15.76	17.49	0.64
MyelinM	5	3.86	3.48	4.11	0.27
G-RatioM	5	0.53	0.50	0.56	0.03
TSJ FCR	8	8.52	7.57	10.64	1.04
V M-M	7	82.4	62.00	96.80	13.21
F M-M	7	26.51	25.03	33.10	2.93
tPk FCR	7	48.00	33.00	65.50	13.28
Pk FCR	7	5.46	2.14	7.76	2.34
Pk FCR/wt	6	57.52	23.45	99.21	32.38
TW/TET	6	0.21	0.09	0.34	0.10
tR/2 FCR	7	109.5	77.0	161.0	34.15
ATw FCR	7	433.0	133.6	792.2	242.01
TwTi FCR	7	3.83	1.51	5.34	1.70
Tet FCR	7	22.88	18.10	25.90	2.48
Tet FCR/wt	6	259.39	200.48	332.99	48.01
Tet/2 FCR	7	20.48	15.40	26.60	4.24
ATet FCR	7	392.2	305.0	566.9	90.10
TeTi FCR	7	19.19	15.50	21.30	2.16
Mass FCR	7	10.30	7.50	14.72	2.53
Wt FCR	7	0.10	0.07	0.14	0.03

Table 9 To show the mean values of the tests used to assess the normal median nerves.

Descriptive Statistics (normal ulnar nerve)

	Valid N	Mean	Minimum	Maximum	Std.Dev.
AxonU	8	8.78	6.70	10.68	1.34
FibreU	7	14.59	11.52	17.15	2.08
MyelinU	7	2.89	2.41	3.36	0.33
G-ratioU	7	0.60	0.58	0.63	0.02
TSJ FCU	9	8.00	6.41	9.72	1.20
V U-U	8	84.82	68.90	100.00	11.83
F U-U	7	25.55	25.10	27.00	0.68
Mass FCU	8	37.59	33.20	47.00	4.30
tPk FCU	4	36.90	31.00	43.00	6.27
Pk FCU	4	2.29	0.66	3.28	1.22
t1/2 FCU	4	106.78	69.50	134.60	28.01
ATw FCU	4	165.9	45.1	309.1	109.20
TwTi FCU	4	1.54	0.44	2.30	0.82
Tet FCU	4	12.65	5.12	18.60	5.70
t1/2 FCU	3	13.20	9.20	18.00	4.45
ATet FCU	3	127.0	36.3	176.1	78.64
TetTi FCU	3	9.17	3.94	14.20	5.13

Table 10 To show the mean values of the tests used to assess the normal ulnar nerves.

RESULTS OF CONVENTIONAL REPAIRS GROUP

The results of the electrophysiological, muscle tension and morphometric tests for the groups of sheep whose median nerves underwent neurotmesis and repair with a method already used in clinical practice are shown. These groups of sheep were:

- Neurotmesis and primary suture group
- Neurotmesis and autologous nerve graft group
- Neurotmesis and conduit (biodegradable glass wrap) repair group

The results for each group are shown by test as mean values with standard error and standard deviation values.

Breakdown Table of Descriptive Statistics

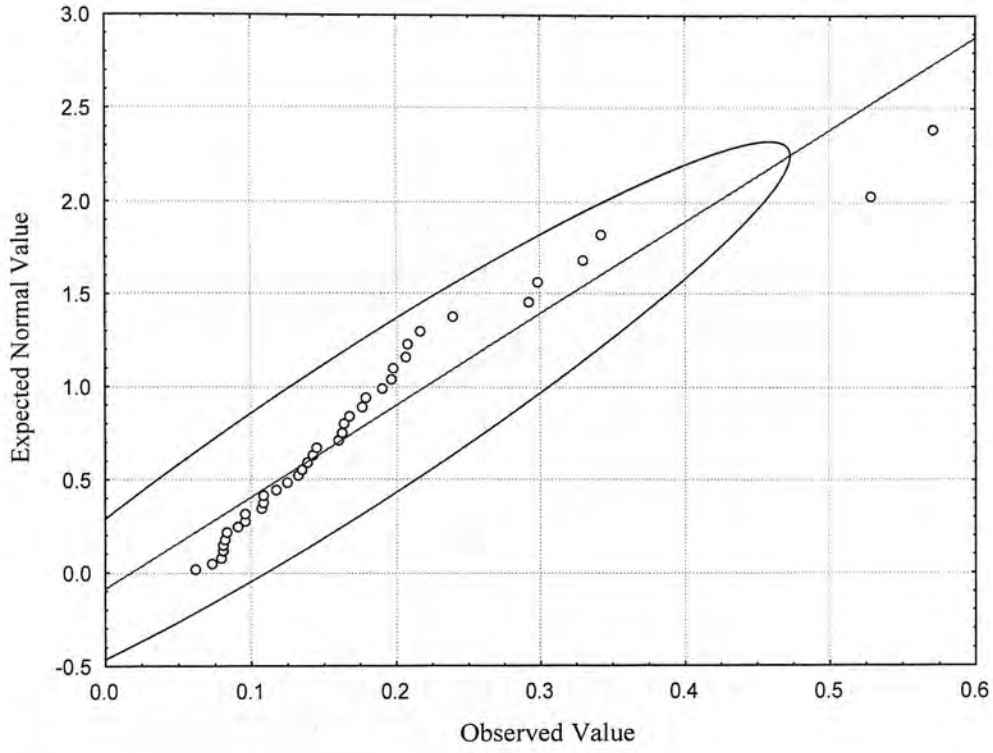
Expt	AxonM Mean	AxonM Number	AxonM Std. dev.	FibreM Mean	FibreM Number	FibreM Std. dev.
NEURO	4.75	4	0.33	8.39	4	0.35
WRAP	4.37	5	0.44	8.49	5	0.52
GRAFT	3.94	2	0.72	6.91	2	0.81
All Grps	4.43	11	0.50	8.17	11	0.77

Expt	MyelinM Mean	MyelinM Number	MyelinM Std. dev.	G-ratioM Mean	G-ratioM Number	G-ratioM Std. dev.
NEURO	4.74	4	0.33	8.39	4	0.34
WRAP	4.37	5	0.44	8.49	5	0.52
GRAFT	3.94	2	0.71	6.91	2	0.81
All Grps	4.43	11	0.50	8.16	11	0.77

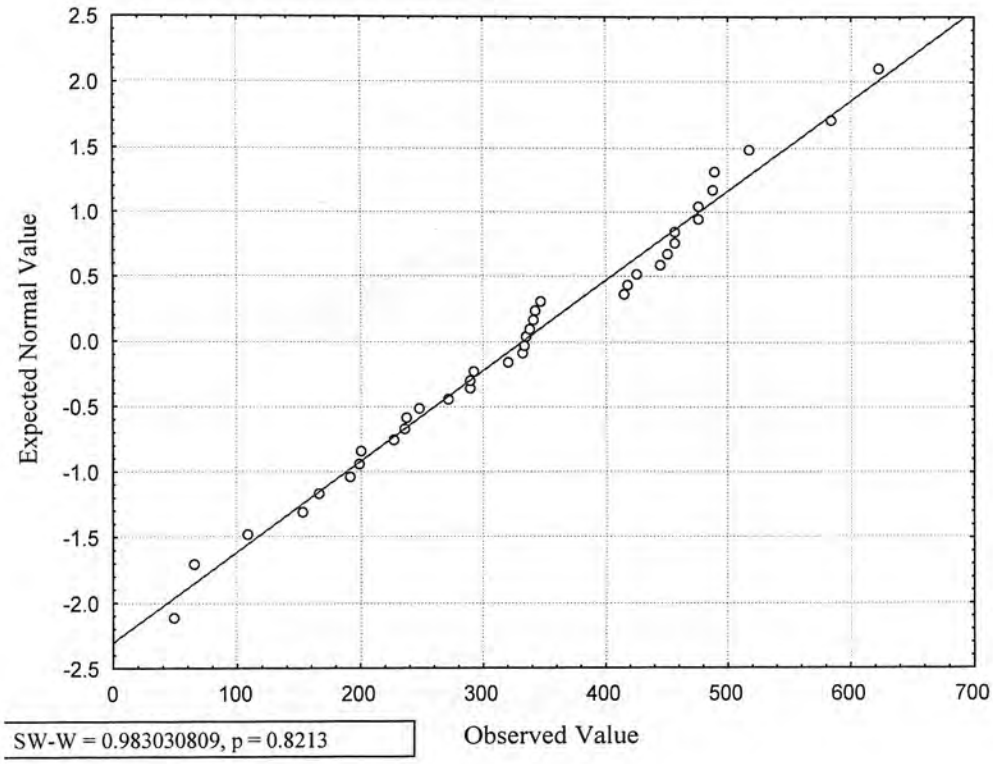
Table 11 To show the means and standard deviation values of the axon and fibre diameters, myelin thickness and G-ratios of the median nerves of the conventional; repair groups of animals.

The table above shows the means and standard deviation values of the axon and fibre diameters, myelin thickness and G-ratios of the median nerves of the conventional repair groups of animals. These variables are presented here as an example to show how the results for each variable were set out.

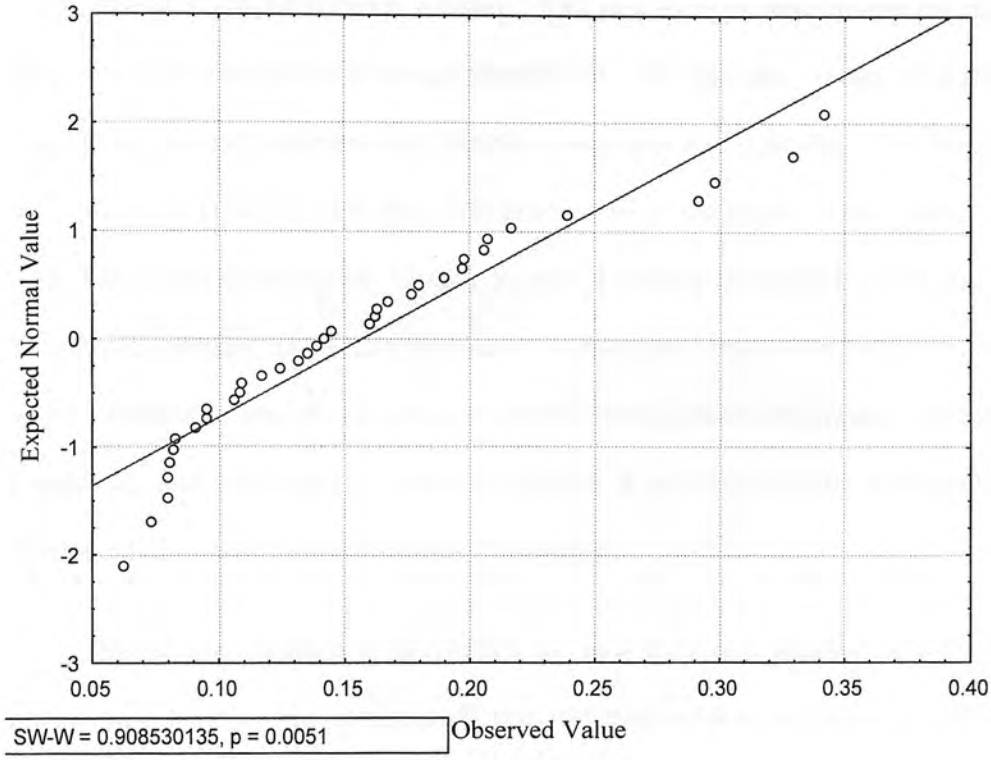
Standard statistical analysis was applied to the results for these groups for all tests (variables) performed. After eliminating outliers in the values for each variable by plotting half normal scatter plots, the variables were tested for normality (to see whether the variables would fit a normal distribution). Examples of the half normal plot with an ellipse added to show outliers and the normal plot with the Shapiro-Wilk test result displayed on the graph are shown on the next pages.



Graph 1 To show an example of the half normal plot with an added ellipse. One was plotted for each variable. The ellipse marks out the outlying results. These were then eliminated from the spreadsheet.



Graph 2 To show an example a normal probability plot of normally distributed data.



Graph 3 An example of a normal probability plot of typically non-parametrically distributed data

One was plotted for each variable. This plot looks at how closely the mean values of each variable fit a normal distribution. On this plot, values of a given variable are normally distributed if they fit closely onto a straight line. The Shapiro-Wilk test result is displayed to the left bottom corner of the graph. If the p value is < 0.05, the values of the given variable are not normally distributed. This data is normally distributed. It was then necessary to employ an F test to look at the variation of the values measured for each test or variable between the three groups. Normally distributed data underwent a one-way ANOVA F test and for the non-normally distributed data the Kruskal-Wallis test was applied.

Significant variation in the values for each measured variable between the groups calculated by the statistical F test was expressed as a probability (p). A probability of <5% (p=<0.05) indicated that there was a 95% chance that there were significant differences in the values for any measured variable between the groups. The Scheffè test was then applied to normally distributed variables that showed between group differences to look at where these differences lay. Non-normally distributed data that showed differences underwent a Mann Witney-U test to look at where differences lay.

Normally distributed results

After applying statistical tests for normality, the variables below were found to be normally distributed.

Myelin, Fibre, Axon, G-ratio: myelin thickness, axon diameter and G-ratio of the median nerves distal to the sites of repair.

TSJ: jitter of the FCR muscles.

V M-M: Maximum conduction velocity of the median nerve across the repair.

Pk/wt: Force produced by a single muscle twitch (N) per unit weight of the FCR muscle (N).

Tet/wt: Force produced by a tetanic stimulus to the FCR muscle (N) per unit weight of it (N).

TW/TET: Ratio of twitch tension to tetanic tension (forces per unit weights of the muscles- N/N).

TeTi: Time tension index of the FCR muscle for the tetanic tension produced.

Mass FCR: Mass of the FCR muscle.

ATet: Area under the tetanic tension trace to half relaxation of the FCR muscle.

Tet/2 and tR/2: Time to half relaxation of the FCR muscles for the tetanic and twitch tensions respectively, from the start of muscle contraction.

An F test, the one-way ANOVA, was applied to look for significant variations in the mean values for each normally distributed variable described, between the groups. This is the analysis of variance. Given a certain number in a group (n), the variance is a function of the sums of (deviation) squares, or SS for short. The results of which are shown below. The within-group variability (SS) is usually referred as the to as Error variance. This term explains that this cannot be accounted for in the current design. The SS Effect can be explained. This is due to the differences in the means between the groups.

Analysis of Variance
Marked effects are significant at $p < 0.05000$

	Error SS	df	Error MS	Effect SS	df	Effect MS	F	p
AxonM	0.89	2	0.44	1.6	8	0.20	2.194	0.173
FibreM	***3.88***	***2***	***1.94***	***2.1***	***8***	***0.27***	***7.329***	***0.015***
MyelinM	***0.49***	***2***	***0.24***	***0.1***	***8***	***0.01***	***16.967***	***0.001***
G-RatioM	***0.01***	***2***	***0.00***	***0.0***	***8***	***0.00***	***4.609***	***0.046***
TSJ FCR	1.48	2	0.74	31.5	15	2.10	0.352	0.708
V M-M	141.28	2	70.64	2084.3	14	148.88	0.474	0.631
TW/TET	0.01	2	0.00	0.1	12	0.00	0.599	0.564
tR/2 FCR	3999.09	2	1999.54	8038.0	13	618.31	3.233	0.072
Tet FCR/wt	14066.45	2	7033.22	353833.4	13	27217.95	0.258	0.776
Tet/2 FCR	101.94	2	50.97	519.4	13	39.96	1.275	0.312
Atet FCR	84438.49	2	42219.24	211383.0	13	16260.23	2.590	0.112
TeTi FCR	131.03	2	65.52	295.7	13	22.75	2.880	0.092
Mass FCR	2.10	2	1.05	36.7	14	2.62	0.399	0.678
Wt FCR (N)	0.00	2	0.00	0.0	14	0.00	0.399	0.678

Table 12 To show the results of the F test performed on the variables shown for the conventional repair groups, that is normally distributed. Significant results are shown in italic bold. SS = sum of the squares, that assesses within groups variation. Df = degrees of freedom, MS = mean of the squares, p = probability and f = numerical value of the statistical test.

The fibre diameter, myelin sheath thickness and the G-ratio values of the groups have shown significant variation between groups. At this level the null hypothesis is rejected.

Scheffé Test
Variable: FibreM
Marked differences are significant at p < .05000

	{1} M=8.39	{2} M=8.49	{3} M=6.91
NEURO {1}		0.957	***0.031***
WRAP {2}	0.957		***0.019***
GRAFT {3}	***0.031***	***0.019***	

Table 13

Scheffé Test
Variable: G-RatioM
Marked differences are significant at p < 0.05000

	{1} M=0.56	{2} M=0.51	{3} M=0.57
NEURO {1}		0.095	0.915
WRAP {2}	0.095		0.104
GRAFT {3}	0.915	0.104	

Table 14

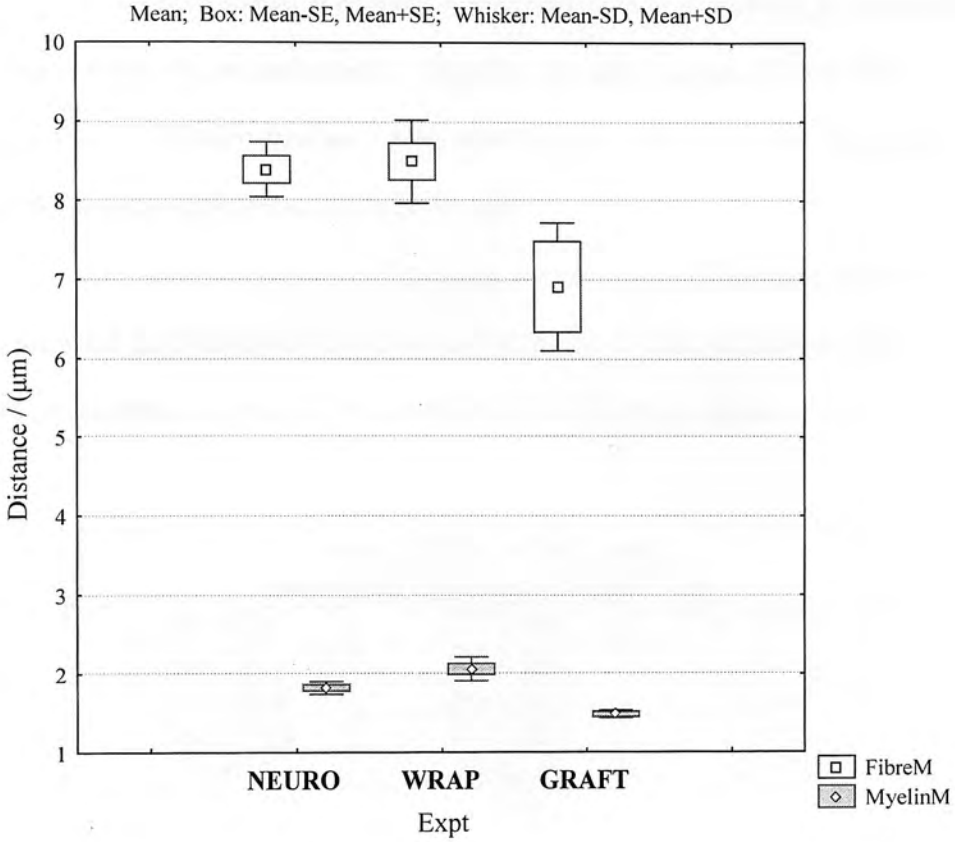
Scheffé Test
Variable: MyelinM
Marked differences are significant at p < 0.05000

	{1} M=1.82	{2} M=2.06	{3} M=1.48
NEURO {1}		0.052	***0.034***
WRAP {2}	0.052		***0.001***
GRAFT {3}	***0.034***	***0.001***	

Table 15 - tables 13,14 and 15 show where the differences for fibre diameter and myelin sheath thicknesses of the conventionally repaired median nerves lay. There are no differences in the means of the G-ratios of the conventionally repaired median nerves. M= mean value for each group.

The G-ratios for the repair groups are not statistically different from one another. There are significant differences for the fibre diameter and the myelin thickness between the graft group and the neurotmesis group and the graft group and the wrap group but not between the neurotmesis and wrap group. The significance of

these differences must be questionable as the graft data contains fewer data points than the other groups (50% less points). The counting of the graft group axonal and fibre diameters was extremely difficult due to the fact that the nerve specimens prepared for histological section were made with a faulty batch of Araldite that did not harden properly when the specimens were set in the moulds. The araldite from these specimens had to be re-dissolved and the specimens re-set in fresh araldite in the specimen moulds. After the araldite had set the blocks of specimen were removed from the mould. These specimens were sectioned on the microtome and prepared on slides in the usual manner. However on observation of the nerve sections through the microscope the nerve fibres appeared irregular and broken up on some sections. It was not possible therefore, to count any axon or fibre diameters on 3 of the graft group sheep's specimens due to this distortion.



Graph 3 To show the differences in the fibre diameter, myelin thickness and G-ratios of the three conventional repair groups.

It has been shown in previous research that the myelin sheath becomes thinner after neurotmesis and nerve repair. The G-ratios of the normal group are not significantly different from any of the repair groups, which mean that the significance of the wrap/graft difference must be in doubt.

The variables shown in the table below were not normally distributed and underwent an F test with the Kruskal-Wallis test to look at the variability of their values between the groups. The results of these tests are as shown.

Kruskal-Wallis ANOVA test Independent (grouping) variable: Expt		
tPk FCR	H =1.000	p =0.607
Pk FCR	H =4.706	p =0.095
ATw FCR	H =5.235	p =0.073
TwTi FCR	H =3.426	p =0.180
Tet FCR	H =6.136	***p =0.047***

Table 16 Kruskal-Wallis test looking for significant differences in non-parametrically distributed data. For these variables a statistical difference was found only in the Tet FCR group (bold italic).

The mean tetanic values for the FCR muscles of the conventional repair groups appear to differ significantly between them. This variable underwent a Mann Witney-U test to look at where between the groups the differences lay. The results are shown below in the table and show that there are significant differences in the mean values of the peak tetanic forces of the wrap group compared to the neurotmesis group but no significant differences between the other groups

MannWitney-U Test for **Tet FCR** By variable: Expt Marked tests are significant at $p < 0.050$			
	NEURO	WRAP	GRAFT
NEURO		***0.007***	0.762
WRAP	***0.007***		0.257
GRAFT	0.762	0.257	

Table 17 To show the where the differences for the tetanic tension Values for the conventional groups lay.

However, these absolute values of tetanic tension do not take into account the weight of the muscle. A more accurate measure is the force per unit weight of the muscles as shown in the Tet FCR/wt column, which has not been shown to be statistically significant.

Breakdown Table of Descriptive Statistics

Expt	Tet FCR Mean	Tet FCR Number	Tet FCR Std.dev.	Tet FCR Std.err.	Tet FCR Minimum	Tet FCR Maximum
NEURO	15.54	6	6.47	2.64	7.30	22.28
WRAP	22.73	6	1.18	0.48	20.47	23.60
GRAFT	17.56	4	6.32	3.16	11.47	25.09
All Grps	18.74	16	5.77	1.44	7.30	25.09

Table 18 To show the means, standard deviations and standard errors, minimum and maximum values of the peak tetanic forces of the conventional repair groups.

From the results there are no real statistical differences between the repair groups and the conventional repair groups. It is therefore more relevant to combine the conventional repair groups into one group for the purposes of statistical analysis when

comparing them with the two end-to-side groups. It was thought that this would make performing statistical tests more practical for the purposes of the statistical computer program and make comparing and understanding results clearer. The new group was termed 'conventional repair'.

It was disappointing that only 2 out of the 6 sections of median nerve taken for histological assessment from the graft group could be counted. This was due to technical problems in the fixing of the specimens, which made it difficult to count axons owing to their distortion. It was likely that axon and fibre diameters would have been underestimated rather than overestimated and would have ordinarily been smaller in the graft group than the primary suture and wrap groups because regenerating axons have to negotiate two suture lines rather than one. As this group was small errors could have occurred during the statistical analysis process of comparing axon and fibre diameters for the three groups of conventional repairs. The myelin sheath thicknesses and G ratios were computed from the axon and fibre diameter values.

With hindsight, the 2 axon and fibre counts from the graft group should have been discarded and not included in the combined 'conventional repair' group. This would have left the size of the conventional group for axon and fibre diameters at 12 rather than 14. This number only represents a small increase in the overall size of such conventional repair groups for axon and fibre diameters leaving the mean values of the groups unlikely to change significantly. It was decided therefore to leave the axon and fibre values for the graft group as part of the combined conventional repair groups.

The validity of the conventional repair group still stands as the idea of comparing end-to-side nerve repair and regeneration to that which may be achieved from any other method of nerve repair already in clinical practice, is the objective of these experiments. It should also be remembered that in every other assessment performed on the nerve repairs from the 3 conventional methods of nerve repair, the results from each group were comparable.

The statistical methods were then reapplied to the data using the conventional repair group as a whole rather than the individual groups of 'wrap', 'graft' and 'neurotmesis' (neuro) experiments.

RESULTS OF THE END-TO-SIDE GROUPS

The results of the end-to-side groups are considered by taking each test in turn that was that was performed on them. The results are compared to the conventional group's results and normal.

The data from the normal group, the end-to-side groups and the conventional repair group underwent statistical analysis. First outliers were rejected and the data was tested for normality using the Shapiro-Wilk test. The variables listed in the table below were seen to be normally distributed. This data included all of the measurements taken for assessment of the ulnar nerve and FCU muscles and the peak forces per unit weights of the FCR muscles and the time tension integral for the FCR muscles. The table shows the results of an F test performed on the normally distributed data to look for significant differences for those variables between the groups of animals. Variables that are significant are shown in bold italic print.

Analysis of variance for normally distributed variables
Marked effects are significant at p < .05000

	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
AxonU	***30.1***	***2***	***15.1***	***46.4***	***26***	***1.79***	***8.427***	***0.002***
FibreU	***58.1***	***2***	***29.1***	***116***	***25***	***4.63***	***6.270***	***0.006***
MyelinU	***2.9***	***2***	***1.45***	***7.2***	***25***	***0.29***	***5.017***	***0.015***
TSJ FCU	1.4	1	1.40	42.7	20	2.13	0.655	0.428
V M-U	0.0	0		3740	11	340	0.000	
V U-U	878	2	438	5350	28	191	2.298	0.119
Pk FCR/wt	2660	3	887	26100	34	768	1.154	0.341
Tet FCR/wt	58290	3	19400	659000	34	19400	1.003	0.403
Atet FCR	136600	3	45000	602000	36	16700	2.724	0.058
Mass FCU	***463***	***2***	***231***	***942.9***	***18***	***52.4***	***4.422***	***0.027***
tPk FCU	2.6	1	2.61	161	5	32.3	0.081	0.788
Pk FCU	0.2	1	0.20	5.3	5	1.06	0.188	0.682
t1/2 FCU	942.0	1	942	6840	5	1370	0.688	0.445
ATw FCU	12100	1	12100	35900	4	9000	1.350	0.310
TwTi FCU	0.1	1	0.10	2.1	4	0.53	0.187	0.688
Tet FCU	5.4	1	5.42	207	5	41.1	0.131	0.732
t1/2 FCU	22.4	1	22.43	60.8	4	15.2	1.475	0.291
ATet FCU	2480	1	2470	13900	4	3500	0.713	0.446
TeTi FCU	0.40	1	0.41	69.5	4	17.4	0.023	0.886

Table 19 To show breakdown and one-way ANOVA testing of the normally distributed data. Significant results are shown in bold italic.

The table shows the F test (one way ANOVA) for this data which shows which variables have statistically significant differences between the experimental groups. The rest of the variables, which include most of the measured variables for the FCR

muscles, underwent the Kruskal-Wallis test. The tables below show the results of these tests.

Kruskal-Wallis ANOVA test Grouping variable: Expt p is significant at <0.05		
AxonM	***H =29.360***	***p =0.000***
FibreM	***H =28.747***	***p =0.000***
MyelinM	H =26.235	p =0.000
G-RatioM	H =6.982	p = 0.073
TSJ FCR	***H =18.477***	***p =0.000***
V M-M	***H =25.089***	***p =0.000***
V U-M	***H =26.552***	***p =0.000***
F M-U	H =0.000	p =1.000
F M-M	H =0.000	p =1.000
tPk FCR	H =6.036	p =0.110
Pk FCR	H =6.755	p =0.081
TW/TET	H =5.013	p =0.171
tR/2 FCR	H =2.720	p =0.437
ATw FCR	H =5.986	p =0.112
TwTI FCR	H =5.584	p =0.134
Tet FCR	H =2.721	p =0.437
Tet/2 FCR	H =3.081	p =0.379
TeTi FCR	H =6.432	p =0.092
Mass FCR	***H =15.882***	***p =0.001***
Wt FCR	***H =15.882***	***p =0.001***

Table 20 To show the results of the non-parametric F test (Kruskal-Wallis) for this data. Significant differences are shown for the mass, weight and TSJ of the FCR muscle and the axon and fibre diameter, myelin thickness and maximum conduction velocity of the median nerve in bold italic (repaired group distal to the repair).

The Kruskal- Wallis test shows the probability of differences between the groups of animals for the various tests performed to assess the nerve repairs and the muscles supplied these nerves. Significant differences are shown for the mass, weight and TSJ of the FCR muscle and the axon and fibre diameter, myelin thickness and maximum conduction velocity of the median nerve. For the experimental groups histological sections were taken distal to the repair sites.

Transcutaneous stimulated jitter (TSJ)

During assessment of the TSJ of the regenerated FCR muscles it was noted how well the muscle was twitching. For the conventional repair groups, good twitch was noted at a minimal stimulation current (3mA). In the graft group however the twitch of the muscle to the naked eye was appreciably less at these small stimulation currents. It was found that up to 20mA was sometimes needed in order to record measurable end-plate action potential spikes on the Medelec screen. In the end-to-side group, for 5 of the FCR muscles, there was no obvious twitch to the naked eye even at stimulation currents of 40-50mA. It is important not to increase the current too much as denervated muscle can eventually be stimulated giving a falsely positive results. When no twitch was seen at 20mA, the muscle was exposed by making a longitudinal incision 10cm proximal to the hoof joint, 1 finger breath medially to the bony part of the forelimb, which is easily palpated through the skin. The FCR muscle is known to lie here from experience with previous normal experiments. The fascia of the muscle was incised longitudinally for 2cm just exposing the muscle. The SFEMG electrode was inserted in this area to assess the jitter for the end-to-side group. This was not necessary for the double end-to-side group as the muscles could be seen to obviously twitching for all muscles in this group. All FCU muscles in all experiments were also easily identified from the twitch.

The TSJ for FCR was found not to be normally distributed ($p= 0.028$) and for the FCU the TSJ is just not normally distributed ($p=0.046$).

Results of the normal group compared with all experimental groups

Using the statistical analysis regime described above the mean values of normal jitter were seen to be statistically significantly different from the jitter from all the experimental groups.

Results of the conventional repairs group and the end-to-side groups

The results for the transcutaneous stimulated jitter for the experimental groups (graft, neurotmesis and wrap) are shown separately and together as the 'conventional repair' group in the tables below. The values of both end-to-side groups are shown for comparison. The mean, maximum and minimum and the standard deviation and error values are shown against normal values.

Expt	TSJ FCR Mean	TSJ FCR Number	TSJ FCR Std.dev.	TSJ FCR Std.err.	TSJ FCR Minimum	TSJ FCR Maximum
E to S	14.72	5	1.30	0.58	13.28	16.50
DE to S	10.87	12	2.17	0.63	7.08	15.81
NEURO	10.23	6	0.72	0.30	8.98	10.99
WRAP	9.55	6	1.34	0.55	7.76	11.51
GRAFT	9.75	6	2.00	0.81	6.28	11.90
MCONT	8.52	8	1.05	0.37	7.57	10.64
All Grps	10.45	43	2.33	0.36	6.28	16.50

Table 21 To show the means and standard deviation and standard error statistics for the stimulated jitter of the FCR muscles for the experimental groups against normal.

Expt	TSJ FCR Mean	TSJ FCR Number	TSJ FCR Std.dev.	TSJ FCR Std.err.	TSJ FCR Minimum	TSJ FCR Maximum
E to S	14.71	5	1.30	0.58	13.28	16.50
DE to S	10.87	12	2.17	0.63	7.08	15.81
MCONT	8.52	8	1.05	0.37	7.57	10.64
CONV	9.85	18	1.39	0.33	6.28	11.90
All Grps	10.45	43	2.33	0.36	6.28	16.50

Table 22 To show the means and standard deviation and standard error statistics for the stimulated jitter of the FCR muscles for the end-to-side experimental groups against the conventional repair groups as a whole against normal.

Kruskal-Wallis ANOVA by Ranks; **TSJ FCR**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H=18.47704, ***p =0.000***

Expt	Valid No.	Sum of the ranks
E to S	5	201
DE to S	12	296
MCONT	8	81
CONV	18	368

Table 23 To show the results of the f test (Kruskal-Wallis) for the stimulated jitter for the experimental groups against normal. The table shows the number of sheep in each group and the p statistic shows that there are significant differences for the jitter between the groups.

The values for the transcutaneous stimulated jitter were found not to be normally distributed (non-parametric) for the experimental groups shown in the table above. To find whether there was any significant variability in the mean values of the 'jitter' between the groups, the data underwent an F test for a non-parametric distribution. This is the Kruskal-Wallis test. The results of this test are shown in the table above and shows that the 'p' value is <0.05 which means it can be said that there is 95% confidence there are statistically significant differences for the jitter

between the groups of sheep. To find out where these differences lay the Mann Witney-U two-sample test was performed with the results shown in the tables below:

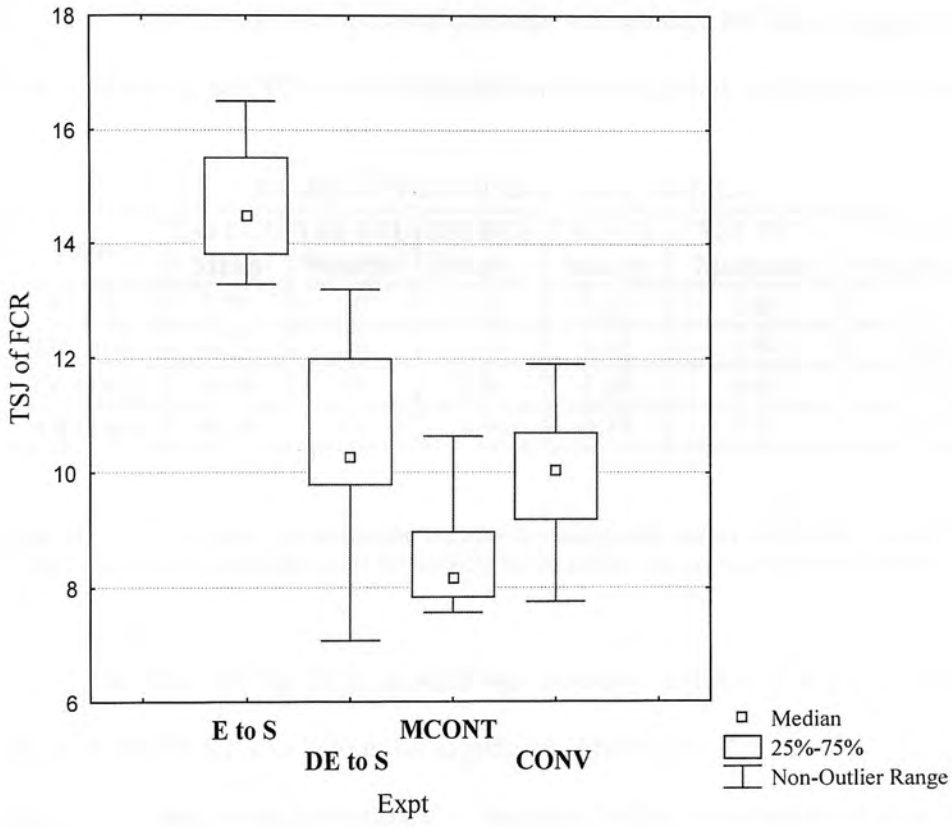
Mann-Whitney U Test (TSJ FCR) Marked tests are significant at p <0.050 By variable: Expt				
	E to S	DE to S	CONV	MCONT
E to S		***0.004***	***0.000***	***0.002***
DE to S	***0.004***		0.305	***0.010***
CONV	***0.000***	0.305		***0.018***
MCONT	***0.002***	***0.010***	***0.018***	

Table 24 To show the results of the Mann Witney-U test that compare the values of the 'jitter' for two groups at once.

The table shows that there are significant differences in the jitter values between the normal groups and the all the other experimental groups, between the end-to-side group and all other groups including the double end-to-side group. The double end-to-side group differed from the normal group but not the conventional repair group. The smallest mean value for the 'jitter' was for the normal group and the highest mean value for the end-to-side group.

In the immature regenerating motor unit there is a larger variability in the time of transmission from stimulation of the motor axon and the recording of the muscle fibres action potential owing to the lower and more variable threshold potentials at the end plate. As the fibre continues to mature, the end plate threshold begins to rise and stabilize reducing the variability (Trontelj, Stålberg, & Mihelin 1990). Measuring the of the jitter value of muscles in rats whose supplying nerves had under gone neurotmesis and subsequent epineurial end-to-end repair showed that at increasing time intervals after the repairs showed, as the muscles regenerated, the jitter values decreased (Lenihan 2000; Lenihan et al. 1997). A high jitter value

suggests therefore, that the neuromuscular junctions of the muscles have not regenerated fully. There is a decreasing trend in jitter values for the different experimental groups towards the lowest value, which is that of the normal group. The box and whisker plot Graph 4 shows this well.



Graph 4 To show the significant differences between the values of the jitter for the end-to-side group compared with normal and the other experimental groups.

Results of the jitter for FCU muscles of the end-to-side groups

The table shows the results of the mean and standard deviation values for the jitter for the FCU and FCR muscles for the end-to-side groups compared to normal.

Breakdown Table of Descriptive Statistics

Expt	TSJ FCU Mean	TSJ FCU Number	TSJ FCU Std.dev.	TSJ FCU Std.err.	TSJ FCU Minimum	TSJ FCU Maximum
E to S	7.71	10	1.35	0.43	5.83	9.61
DE to S	8.21	12	1.54	0.45	5.84	10.23
UCONT	8.00	9	1.20	0.40	6.41	9.72
All Grps	8.00	31	1.36	0.24	5.83	10.23

Table 25 To show the means and standard deviations and number of animals in each group for the transcutaneous stimulated jitter for the FCU for the end-to-side groups compared to normal.

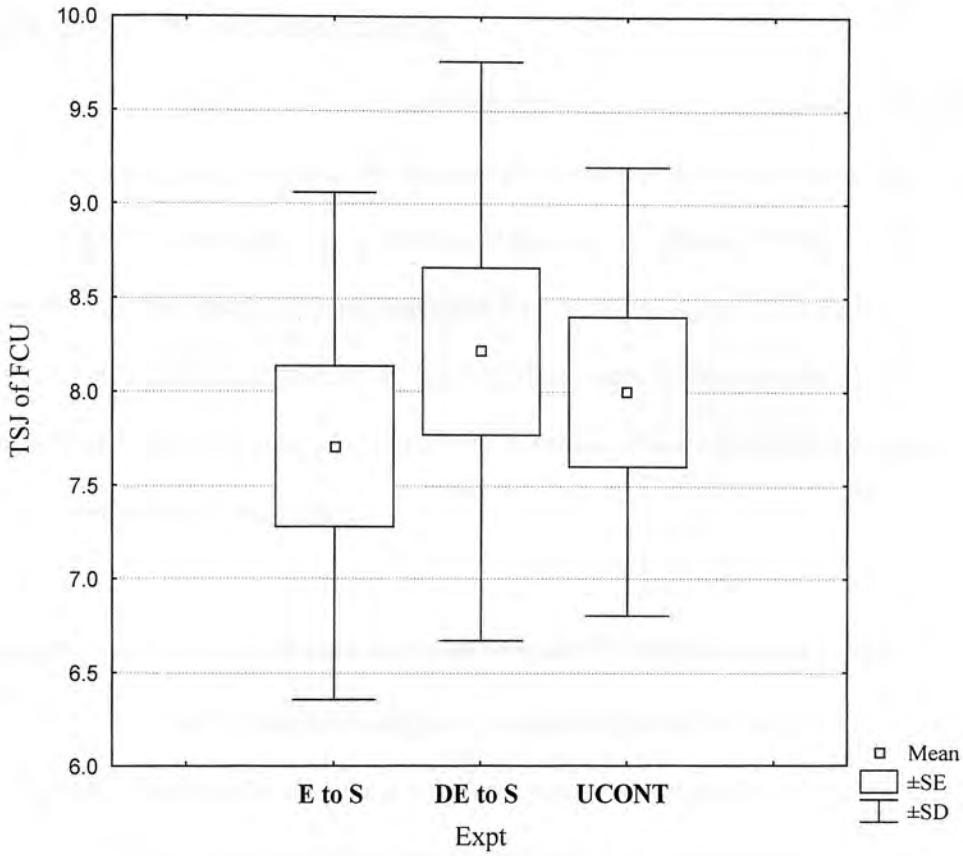
The jitter for the FCU muscles was normally distributed and underwent a one-way ANOVA F test to look for significant differences. This statistical analysis showed that there were no statistical differences for the jitter between both end-to-side groups and the normal group. There is also no difference between the end-to-side groups.

Analysis of Variance: Marked effects are significant at $p < .050$

	SS	df	MS	SS	df	MS	F	p
TSJ FCU	1.40	2	0.70	54.157	28	1.93	0.362	0.700

Table 26 To show the results of the f-test for the jitter of the FCU muscles. There are no statistical differences between the groups of animals as p=0.700.

The jitter values of the FCU muscles for both end-to-side groups are within normal limits of the values for the normal FCU muscles. The results show that either the FCU muscles did not suffer any recordable denervation during the perineurial window process or if the FCU muscles had been partially denervated during this process then the motor end plates of these muscle have been sufficiently reinnervated to give near normal jitter values.



Graph 5 To show the jitter values for the FCU muscles for the end-to-side groups compared to normal. There are no significant differences between the groups. The standard deviations of the mean values from each group are similar.

Maximum conduction velocity

The use of conduction velocity as a means of assessing peripheral nerve function was developed by Berry who showed that the conduction velocity was reduced in regenerating nerve (Berry, Grundfest, & Hinsey 1944). This test is popular as the results are reproducible and easy to obtain. The results for the maximum conduction velocity (CV_{max}) in these experiments will be discussed by taking each group in turn as the end-to-side groups involve more than one pathway due to the nature of the repairs.

Results of all the experimental groups compared with the normal group

After statistical analysis it was shown that all values for the maximum conduction velocity for all the experimental groups were significantly different from normal. These results are shown in the table below. It can be seen by looking at the means of the conduction velocities in table 10 that the normal median nerve maximum conduction velocities are markedly higher than all of the experimental groups.

Sheep	Control	Neuro	Graft	Wrap	E to S (V U-M)	DE to S (V U-M)	DE to S (V M-M)
1	92.6	54.4	63.1	48.6	28.6	17.0	14.4
2	89.5	32.7	32.4	35.6		15.8	33.8
3	68.7	39.5	21.1	46.9	26.3	34.4	28.3
4	98.0	37.8	47.5	46.7	16.0	6.7	26.9
5	77.5	38.0	13.1	43.1	23.5	36.1	38.5
6	62.0	40.0		34.2	17.1	13.7	38.8
7	90.0					18.5	36.3
8	96.8				40.0	40.0	28.3
6						11.9	21.9
10					11.0	23.8	32.1
11						7.3	19.2
12						22.0	23.8
Mean	84.4	40.4	35.4	42.5	23.2	20.0	28.5

Table 27 To show the results of the maximum conduction velocity in milliseconds, achieved across the repaired median nerves in each experimental group compared to the normal conduction velocity of a sheep median nerve. The experimental groups are represented in the table as: Neuro – Neurotmesis and primary suture, Graft – autologous nerve graft of 1cm and Wrap – 2 x 4 cm rectangle of CRG material wrapped around the cut ends of the median nerve. E to S – end-to-side and DE to S – double end-to-side, ulnar to median pathway.

Results of the conventional repairs group compared with the end-to-side groups

The CV_{max} for the conventional repairs have been shown previously not to be significantly different from one another. These have been termed collectively 'conventional repair'.

Breakdown Table of Descriptive Statistics (CV_{max}-repaired median nerve)

Expt	Mean	Number	Std. Dev.	Std. Err.	Minimum	Maximum
NEURO	40.40	6	7.33	2.99	32.70	54.40
WRAP	42.52	6	6.18	2.52	34.20	48.60
GRAFT	35.44	5	20.15	9.01	13.10	63.10
MCONT	84.39	8	13.41	4.74	62.00	98.00
All Grps	53.99	25	24.46	4.89	13.10	98.00

Table 28 To show the means, standard error and deviations and maximum and minimum values for the maximum conduction velocity (CV_{max}) for the repaired median nerves for the conventional repair groups compared to normal.

The end-to-side model is being tested here as an experimental alternative to conventional nerve repair techniques. The double end-to-side model tests whether by attaching the proximal stump of the transected nerve to the donor nerve a distance proximal to the end-to-side neurorraphy site regeneration may be enhanced (conditioning lesion). The CV_{max} from the ulnar to median pathway of both the end-to-side groups' results are compared. The results are shown in the table below with the normal values for reference.

Breakdown Table of Descriptive Statistics
(CV_{max} - repaired median nerves)

Expt	V U-M Mean	V U-M Number	V U-M Std. dev.	V U-M Std. dev.	V U-M Minimum	V U-M Maximum
E to S	23.21	7	9.65	3.65	11.00	40.00
DE to S	20.60	12	11.09	3.20	6.70	40.00
MCONT	82.44	7	13.21	4.99	62.00	96.80
CONV	39.69	17	11.79	2.86	13.10	63.10
All Grps	38.64	43	24.01	3.66	6.70	96.80

Table 29 To show the means and standard deviations and errors and minimum and maximum values for the maximum conduction velocities of both end-to-side groups against that of the conventional repairs and normal values. The table shows the conventional repairs as as one group (CONV). E to S and DE to S = end-to-side suture.

Breakdown Table of Descriptive Statistics
(CV_{max} - repaired median nerves)

Expt	V M-M Mean	V M-M Number	V M-M Std. dev.	V M-M Std. err.	V M-M Minimum	V M-M Maximum
E to S	23.21	7	9.65	3.65	11.00	40.00
DE to S	28.53	12	7.76	2.24	14.40	38.80
MCONT	82.44	7	13.21	4.99	62.00	96.80
CONV	39.69	17	11.79	2.86	13.10	63.10
All Grps	40.85	43	22.16	3.38	11.00	96.80

Table 30 To show the means and standard deviations and errors and minimum and maximum values for the maximum conduction velocities of both end-to-side groups against that of the conventional repairs and normal values. In this table V M-M is the mean value of the CV_{max} across the median nerve repairs and for the double end-to-side group this is the 'bridge' repair.

V U-M was not normally distributed and underwent an F test for non-parametric data (Kruskal-Wallis). This was to assess the variability of the mean values of the CV_{max} of the repaired median nerves between the experimental groups and normal where V U-M for the double end-to-side group is the CV_{max} for the end-to-side pathway.

Kruskal-Wallis ANOVA by Ranks, **V U-M**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H = 26.55241,
p =0.0000

	Valid N	**Sum of Ranks**
E to S	7	96.00
DE to S	12	140.00
MCONT	7	279.00
CONV	17	431.00

Table 31 To show that there are significant differences in the mean values of the CV_{max} of the repaired median nerves where V U-M for the double end-to-side group is the CV_{max} across the end-to-side repair.

The table shows that the mean CV_{max} in the double end-to-side group is smaller than that of the end-to-side group. Even though this difference is not statistically significant it gives the impression that end-to-side regeneration of nerve fibres was better in the end-to-side group than the double end-to-side. Out of the 11 sheep in the end-to-side group, we were only able to get a reading for the CV_{max} in 7 of these animal's FCR muscles. In the double end-to-side group the ability to obtain conduction velocities was more consistent.

These 4 muscles in the end-to-side group looked extremely degenerate to the naked eye. 3 muscles did not twitch at all with up to 50mA current stimulation to the median nerve distal to the neurorraphy site. Even direct stimulation of the muscle did not produce a twitch. In the other muscle a very small twitch could be seen with the naked eye, with 55mA of current stimulation to the nerve. 3 of the FCR muscles in the end-to-side group where a reading could be obtained were achieved only with a current of 25-30mA applied to the nerve. Twitches of the FCR muscles and adequate CV_{max} readings in the double end-to side group were easily achieved with

up to 20mA of current. The possible problems here lie in the fact that at higher stimulation currents, other musculature in the field begins to twitch as well as the FCR muscle. This makes it difficult to record a discrete CMAP of the FCR muscle for conduction velocity measurement. This may have made some of the MCV recordings for the end-to-side group inaccurate.

Mann-Whitney U Test (CV_{max}) Marked tests are significant at p <0.050 By variable: Expt					
	E to S	DE to S	CONV	BRIDGE	MCONT
E to S		0.482	***0.005***		***0.001***
DE to S	0.482		***0.001***		***0.000***
CONV	***0.005***	***0.001***		***0.006***	***0.000***
BRIDGE	0.261		***0.006***		***0.000***
MCONT	***0.001***	***0.000***	***0.000***	***0.000***	

Table 32 To show Mann Witney-U tests comparing the end-to-side pathways in both end-to-side groups (V U-M), and comparing end-to-side to the bridge technique (V M-M). Again these are compared to the conventional repair techniques and normal. Significant differences between two groups are shown in bold italic.

Statistical analysis shows that the CV_{max} of the end-to-side groups were not significantly different from each other. However these groups were significantly different from the conventional repairs and normal. The conventional repairs were also significantly different from normal.

Results of the conventional repairs group compared with the end-to-side and 'bridge' repairs

The second pathway of axonal regeneration to be considered in the double end-to-side group is the median-to-median path (V M-M). The direction of axonal growth has been hypothesised to be from the proximal neurorraphy of the transected median nerve in this group, through the donor ulnar nerve 'bridge' to the distal median to ulnar neurrorrhaphy. In fact, this is in effect another form of conduit as the epineurium or perineurium (due to the window made) could act as guide for regenerating axons. It probably cannot be assumed that collateral axons from the donor ulnar nerve would sprout from the proximal end-to-side neurorraphy site, grow

towards the distal end-to-side neurorraphy site and form or grow into already established collateral branches into the distal stump of the attached median nerve. There is no evidence as yet to support this theory. The results of the CV_{max} for this pathway are shown in the table below with end-to-side (ulnar to median) neurorraphy CV_{max} and CV_{max} conventional repair groups.

Breakdown Table of Descriptive Statistics (E to S and DE to S -'bridge')

Expt	V M-M Mean	V M-M Number	V M-M Std.dev.	V M-M Std.err.	V M-M Minimum	V M-M Maximum
E to S	23.21	7	9.65	3.65	11.00	40.00
DE to S	28.52	12	7.76	2.24	14.40	38.80
MCONT	84.39	8	13.41	4.74	62.00	98.00
CONV	39.69	17	11.79	2.86	13.10	63.10
All Grps	42.15	44	23.54	3.55	11.00	98.00

Table 33 To show the means and standard deviations and errors and minimum and maximum values for the maximum conduction velocities of the repaired pathways of the median nerve in the experimental groups shown. The first table the shows the conventional repairs as separate groups and the second table shows these repairs as one group (CONV). E to S =end-to-side suture and DE to S = 'bridge' or double end-to-side suture.

The average CV_{max} is higher for the 'bridge' technique (of the double end-to-sides) than in end-to-side neurorraphy (E to S).

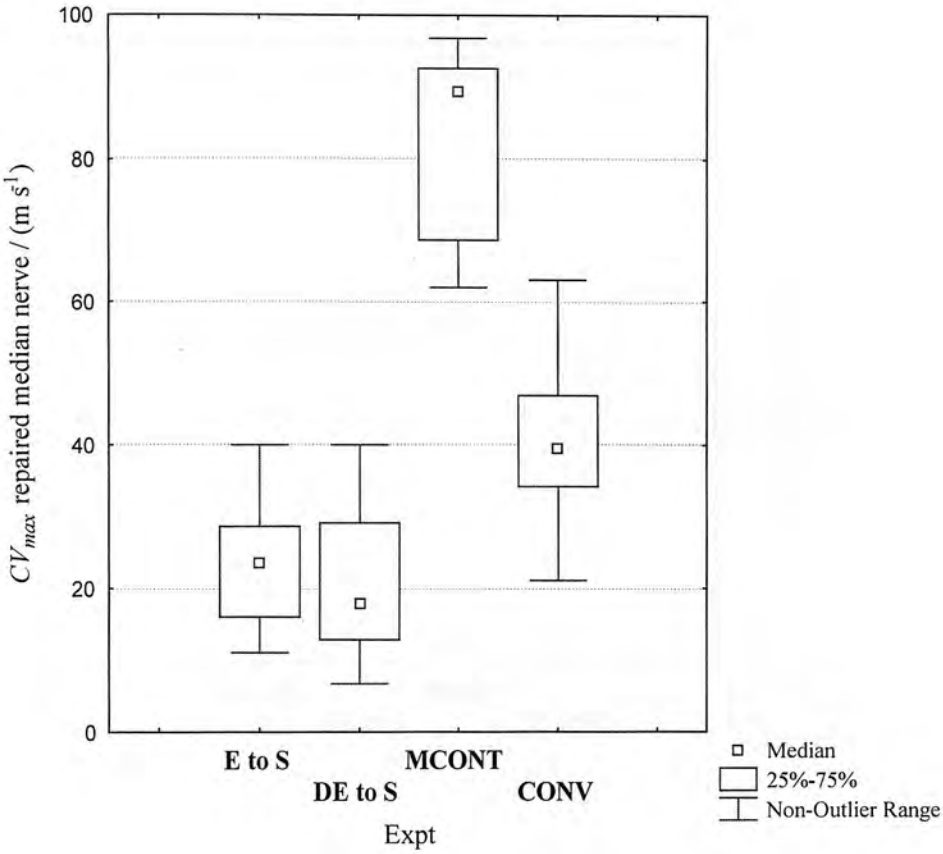
Kruskal-Wallis ANOVA by Ranks;
V M-M
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =25.08628,
p =0.0000

	Valid N	Sum of Ranks
E to S	7	73.50
DE to S	12	174.00
MCONT	7	279.00
CONV	17	419.50

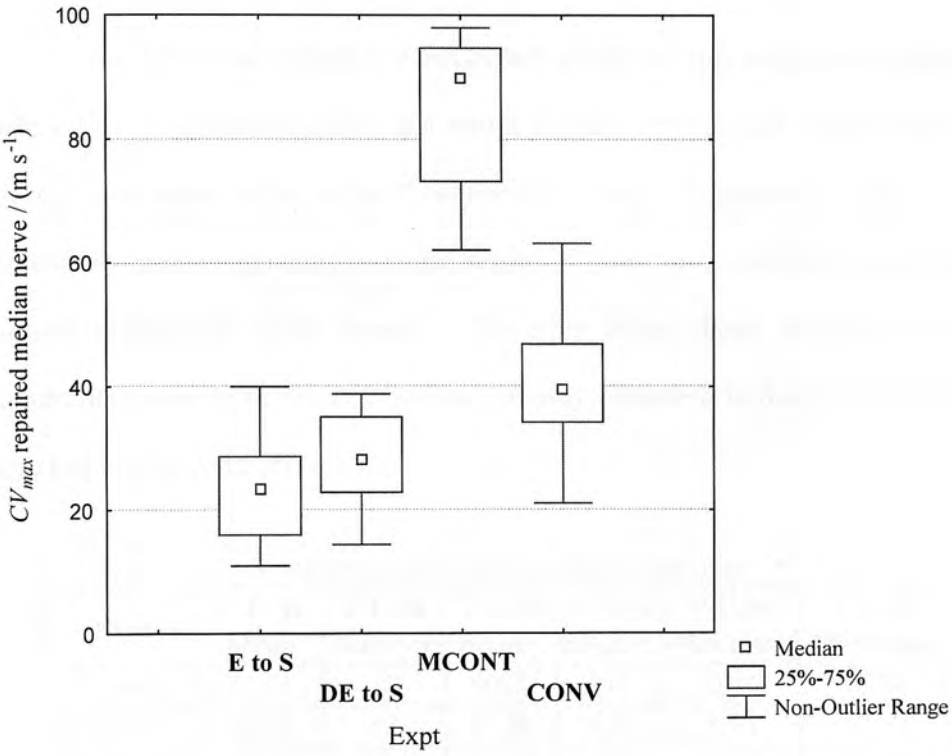
Table 34 To show that there are significant differences in the mean values of the CV_{max} of the repaired median nerves where V M-M for the double end-to-side group is the CV_{max} across the 'bridge' repair.

However, statistical analysis shows that the CV_{max} of the end-to-side and bridge techniques were not significantly different from each other. However these groups were significantly different from the conventional repairs and normal. The conventional repairs were also significantly different from normal. The box and whisker plots show pictorial representation of these results.

We also looked at the conduction velocity of the median to ulnar pathway of the double end-side group but the results may show some inaccuracies due to the fact the ulnar nerve below the level of the double neurorraphy had to be cut so a discrete CMAP trace of the more important ulnar to median pathway could be recorded without interference from CMAPs from other muscles supplied by the ulnar nerve.



Graph 6 To compare the maximum conduction velocity of the median nerve repairs for the different experiments and normal group, where DE to S is the end-to-side pathway.



Graph 7 To compare the maximum conduction velocity of the median nerve repairs for the different experiments and normal group, where DE of S is the 'bridge' pathway.

Comparison of the CV_{max} of the end-to-side and bridge pathways

The double neurorraphy of end-to-side group not only introduces an end-to-side path for regenerating axons but also a possible conduit path of the section of donor ulnar nerve between the 2 neurorraphy sites. Regenerating axons could potentially use the epineurium of this section of nerve as a scaffold to guide their growth towards the distal stump. The table below shows the values of the conduction velocity across this 'bridge' pathway compared to those of the end-to-side and conventional groups.

Breakdown Table of Descriptive Statistics

Expt	V U-M Mean	V U-M Number	V U-M Std.dev.	V U-M Std.err.	V U-M Minimum	V U-M Maximum
E to S	23.21	7	9.65	3.65	11.00	40.00
DE to S	20.60	12	11.09	3.20	6.70	40.00
CONV	39.69	17	11.79	2.86	13.10	63.10
BRIDGE	28.52	12	7.76	2.24	14.40	38.80
All Grps	29.72	48	12.88	1.86	6.70	63.10

Table 35 To show the means, standard error and deviation values of the *CVmax* for the experimental groups where the double end-to-side group pathway is the 'bridge'.

The conduction velocity for the 'bridge' pathway was not normally distributed and the mean values of this variable for all the groups in the table above were tested statistically to look for significant variation of them between the groups. This is the Kruskal-Wallis test, the results of which are shown in the table below:

Kruskal-Wallis ANOVA by Ranks
CVmax
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =16.78185, **p =0.001**

	Valid Number	**Sum of Ranks**
E to S	7	124.00
DE to S	12	179.50
CONV	17	594.00
BRIDGE	12	278.50

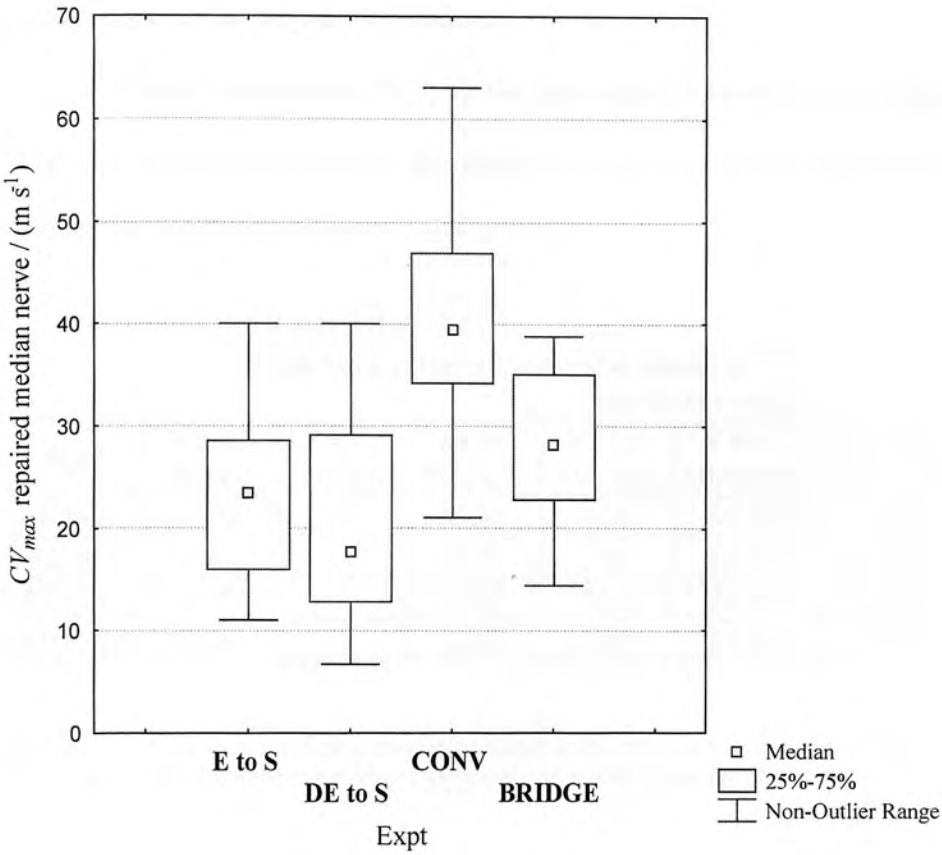
Table 36 To show the results of the Kruskal Wallis F test to look for significant variation of the mean values of the *CVmax* between the groups. The p value is in bold type and show there are significant inter-group differences.

The F test shows there are significant differences for the mean values of the *CVmax* between the groups. The Mann Witney-U test showed where between the groups these differences lay.

Median nerve repair: *CVmax* (ms^{-1}) Mann Witney-U test				
	E to S	DE to S	CONV	BRIDGE
E to S		0.482	***0.005***	0.261
DE to S	0.482		***0.001***	0.060
CONV	***0.005***	***0.001***		***0.005***
BRIDGE	0.261	0.060	***0.005***	
Mean	**23.10**	**20.60**	**39.69**	**28.52**

Table 37 To show the results of the Mann Witney-U test that looks at where between the groups the differences of the mean values of the *CVmax* lie.

There are differences in the *CVmax* for these groups between the conventional repair groups and the end-to-side and bridge groups. There are no differences between the end-to-side and bridge groups.



Graph 8 To compare the differences of the CV_{max} for the conventional repair groups to that of the end-to-side and bridge pathways.

Results of the CV_{max} for the ulnar nerves

The mean values of the CV_{max} for the ulnar nerves were normally distributed. The groups of animals involved in this analysis are the normal ulnar group, the end-to-side group and the double end-to-side group.

Breakdown Table of Descriptive Statistics
CV_{max}, ulnar nerves (ms)

Expt	V U-U Mean	V U-U Number	V U-U Std.dev.	V U-U Std.err.	V U-U Minimum	V U-U Maximum
E to S	83.02	10	12.46	3.94	68.60	101.30
DE to S	70.74	11	10.21	3.80	56.50	93.80
UCONT	84.83	8	11.83	4.18	68.90	100.00
All Grps	78.86	29	12.82	2.38	56.50	101.30

Table 38 To show the means, standard deviations and errors, minimum and maximum values of the CV_{max} of the normal ulnar nerves and those that underwent the making of a perineurial window.

The values were tested for variability between the groups by using the one-way ANOVA F test. The results are displayed below:

Analysis of Variance: Marked effects are significant at p < .05000

	SS	df	MS	SS	df	MS	F	p
V U-U	1033.31	2	516.66	3048.47	25	121.93	4.237	***0.026***

Table 39 To show that there is significant variability in the means of the CV_{max} for the ulnar nerves between the groups

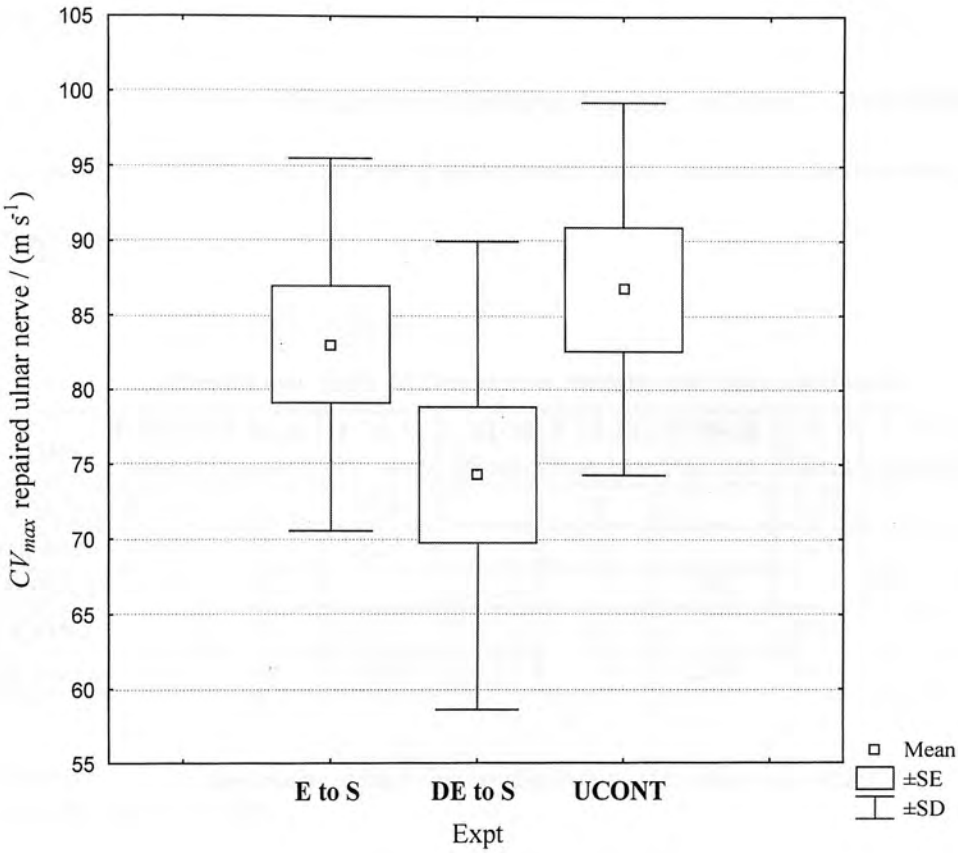
The Scheffé test was then applied to look for where between the groups of animals the differences lay:

Scheffé Test
Variable: **V U-U**
Marked differences are significant at $p < 0.05000$

	{1}	{2}	{3}
E to S {1}		0.139	0.776
DE to S {2}	0.139		***0.037***
UCONT {3}	0.776	***0.037***	

Table 40 To show where between the groups the differences in the means for the CV_{max} lie.

The Scheffé *post-hoc* test shows that are significant differences in the means of the CV_{max} between the double end-to-side and the normal group but no differences between the end-to-side group and the normal group. There were no technical problems in measuring the conduction velocities for these nerves although it was sometimes difficult to achieve a clear take-off point on the CMAP trace for the FCU muscle due the double spiked appearance of it. This appearance was probably due to the bi-pennate nature of the muscle and its very close proximity to other muscles that may have been stimulated simultaneously. The box and whisker plot below shows these differences.



Graph 9 To compare the CV_{max} of the ulnar nerve for the end-to-side groups and the normal group.

F-wave

F-wave studies were performed on the end-to-side pathways in both of these groups. The results were compared to normal F wave studies for the median and ulnar nerves.

Breakdown Table of Descriptive Statistics: (F wave pathways)

Expt	F M-U Mean	F M-U Number	F M-U Std. dev.	F M-M Mean	F M-M Number	F M-M Std. dev.	F U-U Mean	F U-U Number	F U-U Std.dev.
E to S	35.90	7	8.06		0		40.08	5	15.98
DE to S	30.38	12	5.21		0		35.36	8	15.91
MCONT		0		26.51	7	2.93		0	
UCONT		0			0		25.56	7	0.68
All Grps	32.41	19	6.76	26.51	7	2.93	33.11	20	13.53

Table 41 To show results of the F wave studies in the end-to-side groups against normal for the median and ulnar nerves.

Statistical analysis performed on the F wave data showed no significant differences between the end-to-side groups or compared to normal median or ulnar nerves. All these results show are that an F wave response can be achieved in the end-to-side groups with similar latencies to that which can be achieved in normal nerves. It can be said therefore, that impulses are being successfully conducted across the end-to-side neurorrhapy sites meaning that nerve regeneration has occurred as these sites.

Twitch tension

To assess the physiology of the recovered muscles twitch and tetanic tension tests were performed on them. The FCR muscles of the conventional repairs group were assessed to compare these types of nerve repairs to end-to-side and 'bridge' type nerve repairs.

Results of the twitch tension of the conventional repairs group compared with the normal group

	Pk (N)	tR/2 (ms)	ATw (mNs)	TwTi (N)	Mass (g)
MCONT	5.45	109.50	433.01	3.83	10.30
NEURO	2.60	86.50	136.64	2.19	5.15
GRAFT	2.06	72.88	97.64	1.35	5.88
WRAP	4.87	111.83	387.58	3.22	5.89

Table 42 Mean values of the twitch tension results of the FCR muscle in the experimental groups and the normal median group.

It has been found after statistical analysis that there are no significant differences for the above twitch tension measurements between the wrap, graft or neurotmesis groups. These groups can be added together for the purposes of comparison to normal groups and the end-to-side groups.

Results of the twitch tension of the end-to-side groups compared to the conventional repairs and normal groups – FCR muscle

The following table shows the mean values with the standard deviation for the measurements taken during twitch tension studies for the end-to-side groups, conventional repair group and the normal group.

Breakdown of statistics of the twitch tension data for the FCR muscles of the repaired median nerves (V U-M)

Expt	tR/2 FCR Mean	tR/2 FCR Number	tR/2 FCR Std.dev.	ATw FCR Mean	ATw FCR Number	ATw FCR Std.dev.
E to S	79.20	5	25.05	174.65	5	117.72
DE to S	92.21	12	18.77	215.31	12	118.13
MCONT	109.50	7	34.15	433.01	7	242.02
CONV	92.59	16	28.33	221.00	16	229.01
All Grps	93.76	40	26.92	250.60	40	204.93

Table 43

Expt	tPk FCR Mean	tPk FCR Number	tPk FCR Std.dev.	Pk FCR Mean	Pk FCR Number	Pk FCR Std.dev.
E to S	46.90	5	5.54	2.29	5	1.21
DE to S	38.08	12	5.75	3.18	12	1.80
MCONT	48.00	7	13.30	5.47	7	2.34
CONV	45.47	16	17.34	3.32	16	2.79
All Grps	43.88	40	13.07	3.52	40	2.41

Table 44

Expt	TwTi FCR Mean	TwTi FCR Number	TwTi FCR Std.dev.	Pk FCR/wt Mean	Pk FCR/wt Number	Pk FCR/wt Std.dev.
E to S	1.59	5	0.87	34.21	5	11.07
DE to S	2.23	12	1.22	60.83	12	31.15
MCONT	3.83	7	1.70	57.52	6	32.38
CONV	2.36	16	1.87	51.40	15	26.36
All Grps	2.48	40	1.66	53.08	38	27.90

Table 45

Tables 43,44 and 45 show means and standard deviation statistics and number in each group of animals for the twitch tension data. DE to S is the end-side pathway.

The force per unit weight of the FCR was the only variable in the twitch data that was normally distributed. This variable underwent the ANOVA F test and no significant variation was found in their means for this value between the groups. This is shown in the table below.

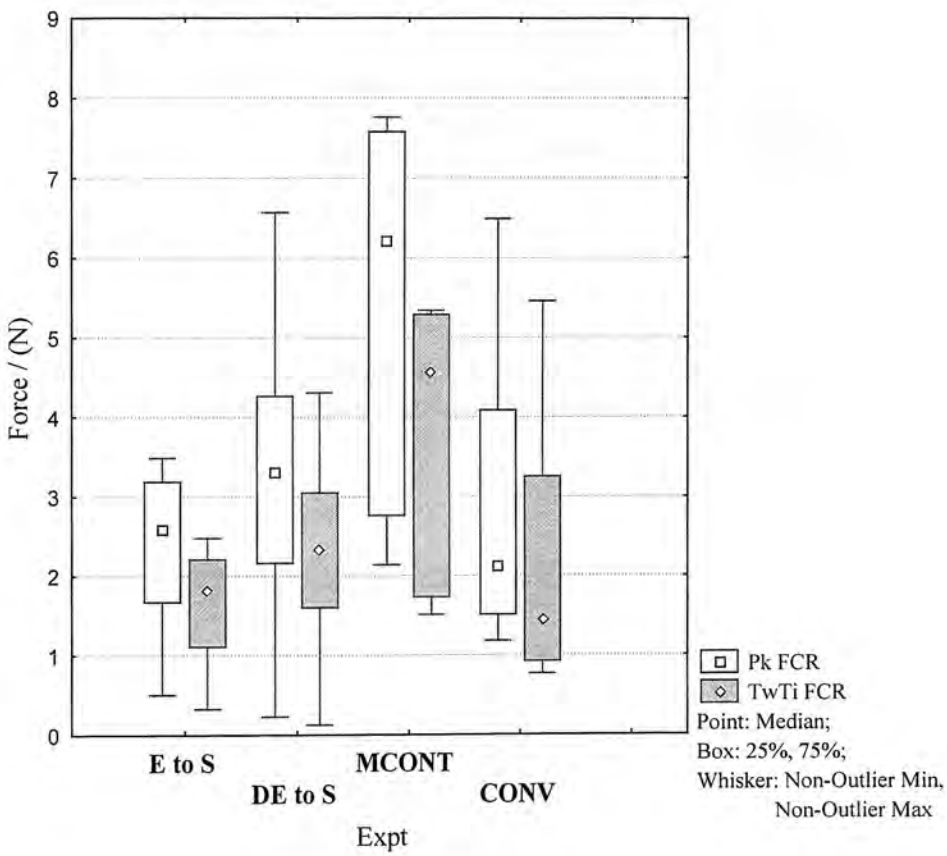
Analysis of Variance: Marked effects are significant at $p < .050$

	SS	df	MS	SS	df	MS	F	P
Pk FCR/wt	2661.65	3	887.22	26133.01	34	768.61	1.154	0.341

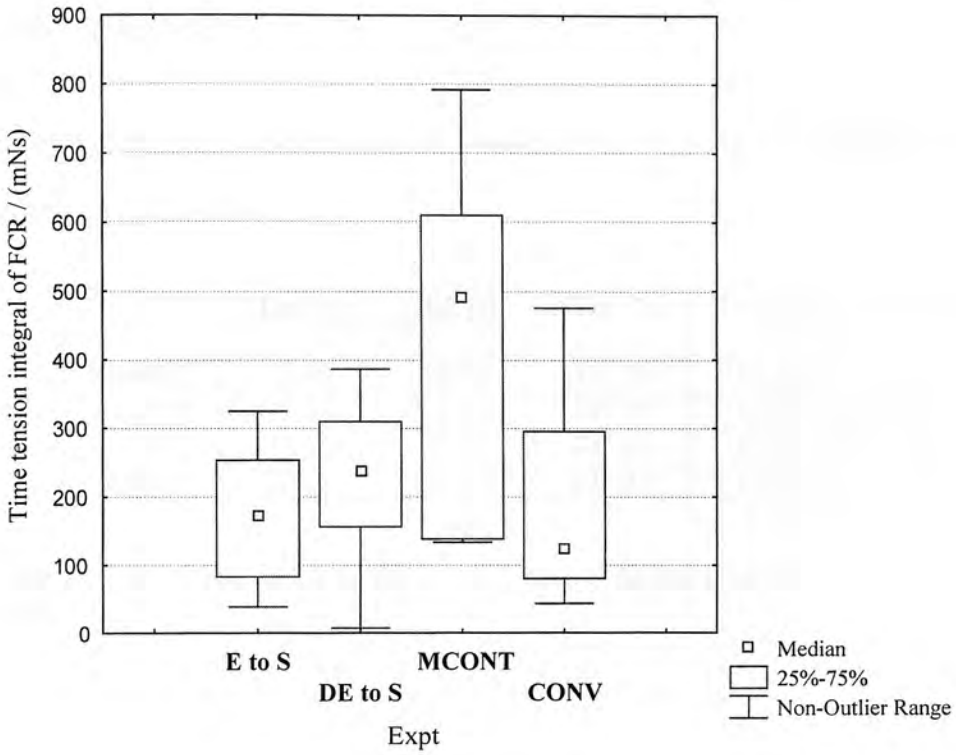
Table 46 To show that there are no significant differences between the experimental groups and the normal group for peak force per unit weight of the FCR muscle.

All the other measurements for the twitch tension tests shown above in the table of means were not normally distributed and underwent the Kruskal-Wallis test. None of this data showed significant differences between the groups.

However the mean values of the of the peak twitch, the time tension integral and time tension index for the normal FCR muscles are nearly twice those of the experimental groups with the lowest mean values for these variables for the end-to-side group. This result fits with previous work that has shown that muscles have reduced contractile properties after nerve repair. The box and whisker plots below compare these variables between the groups.



Graph 10 To show the distribution of values for the peak force of contraction for the muscle twitch and the time tension index (N).



Graph 11 To show the distribution of values for the time tension integral for the FCR muscles of the experimental groups and normal muscles (mNs).

Tetanic tension

Results from the measurement of tetanic tension for the conventional repairs group – FCR muscles

	Tet (N)	R/2 (s)	ATet (Ns)	TeTi (N)	Mass (g)
MCONT	22.89	20.49	392.20	19.19	10.30
NEURO	15.54	13.81	197.56	11.82	5.15
GRAFT	17.56	12.95	165.21	11.95	5.88
WRAP	22.73	18.63	332.43	17.78	5.89

Table 47 Mean values of the tetanic tension results in the experimental groups and the normal group.

Results of the tetanic tensions of the end-to-side repair groups compared with the conventional repairs and normal FCR muscles

The means and standard deviation statistics and number in each group of animals for the tetanic tension data for each group are shown in the table below.

Breakdown of statistics for the tetanic tension data for the FCR muscle						
Expt	**Tet FCR Mean**	**Tet FCR Number**	**TetFCR Std.dev.**	**Tet FCR/wt Mean**	**TetFCR/wt Number**	**TetFCR/wt Std.dev.**
E to S	19.08	5	7.05	304.63	5	42.07
DE to S	20.48	12	15.05	330.46	11	164.95
MCONT	22.89	7	2.48	259.39	6	48.01
CONV	18.74	16	5.77	369.83	16	156.61
All Grps	20.03	40	9.22	332.41	38	139.20
Expt	**Tet/2 FCR Mean**	**Tet/2 FCR Number**	**Tet/2 FCR Std.dev.**	**ATet FCR Mean**	**ATet FCR Number**	**ATet FCR Std.dev.**
E to S	15.52	5	3.89	224.37	5	108.34
DE to S	19.98	12	11.39	244.26	12	138.26
MCONT	20.49	7	4.24	392.20	7	90.10
CONV	15.40	16	6.44	240.05	16	140.43
All Grps	17.68	40	7.91	266.00	40	137.59

Tables 48 and 49

Expt	**TeTi FCR Mean**	**TeTi FCR Number**	**TeTi FCR Std.dev.**
E to S	14.40	5	5.56
DE to S	13.77	12	7.06
MCONT	19.19	7	2.16
CONV	14.09	16	5.33
All Grps	14.92	40	5.73

Table 50. Tables 48,49 and 50 show means and standard deviation statistics and number in each group of animals for the tetanic tension data.

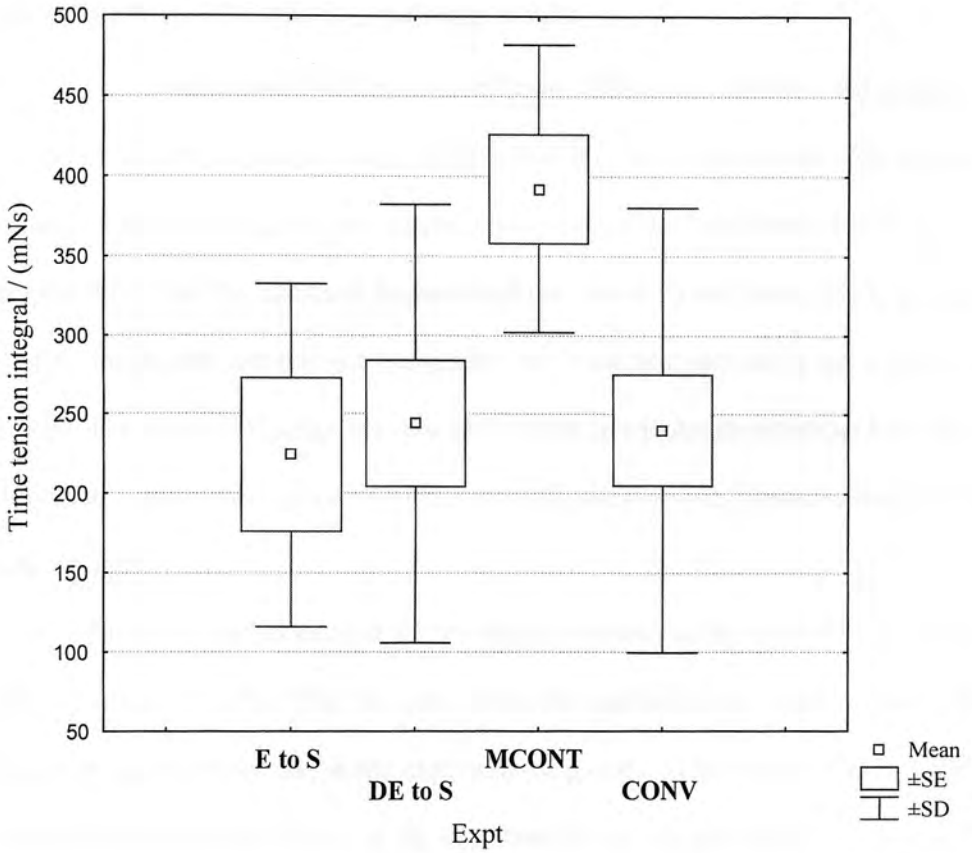
Analysis of Variance: Marked effects are significant at $p < .050$

	SS	df	MS	SS	df	MS	F	p
Tet FCR/wt	58290.6	3	19430.22	658583.3	34	19370.10	1.003	0.403
ATET FCR	136600.2	3	45533.39	601748.3	36	16715.23	2.724	0.058

Table 51 To show that there are no significant differences between the experimental groups and the normal group for the tetanic force per unit weight of the FCR muscle and the area under the tetanic tension curves.

The F test for the normally distributed data of the tetanic tension experiments showed there were no real statistical differences between the groups for the above variables. However the area under the tetanic tension curves showed that the normal FCR tetanic tensions differed considerably from normal, with the normal FCR tetanic tensions being higher. This is shown quite well in the box and whisker plot below.

The peak tetanic force in newtons, time to half peak and the time tension index for the tetanus were not normally distributed and the Kruskal-Wallis test was performed which did not show any differences for these variables between the groups.



Graph 12 Comparison of the areas of the tetanic tension curves (time tension integral) for the experimental groups with normal.

Interpretation of the muscle physiology results

There were found to be no significant differences between the groups or compared to normal group for any of the twitch tension variables although the mean values for the normal group are nearly twice those of the experimental groups. The graphs show that the standard deviation of the values of the peak twitch and time tension index and integral for the groups are wide but less so in the end-to-side group. The number of animals in the end-to-side group though are about half that of the other experimental groups which may make these results less accurate than the other groups.

The time tension integral for the tetanic tensions of the normal FCR muscles had a p value of 0.058. The box plot shows the standard error of the normal group does not overlap with that of the experimental groups. The means of this group is 392 mNs whereas the means of the experimental groups lie between 224 and 244 mNs. The mean values of the experimental groups are very similar with a relatively small standard deviation. The size of the normal group compared to the conventional and DE to S group is nearly half however, which perhaps makes the difference between the mean for the normal misleadingly high compared to the experimental groups. After denervation and subsequent nerve repair and reinnervation, it cannot be said in practical terms that in all cases muscles regain the strength they had before nerve injury. These results for the twitch and tetanic force data may actually be correct even though they are not significant.

FCU muscle physiology

In this work it was not possible to measure any muscle physiology data for the FCU muscles of the double end-to-side group. The reasons for this are as follows:

The electrophysiological experiments and muscle physiology were performed on the end-to-side pathways first before the donor nerve and muscles were assessed. It was necessary for the ulnar nerve distal to the end-to-side neurorraphy site (distal site in the double end-to-side pathway) to be transected in order to achieve a measurable CMAP when measuring the CV_{max} of the ulnar to median end-to-side pathways.

Unfortunately, there was no solution to this problem. It was not really feasible to perform the muscle experiments before the electrophysiological experiments because in order to measure the twitch and tetanic forces the muscles have to be detached at their tendinous insertions. This would make subsequent measuring of jitter values and achieving a measurable CMAP for CV_{max} measurement extremely difficult and yielding potentially inaccurate results. It was felt that the jitter and CV_{max} experiments provide a more consistent assessment of the degree of nerve and muscle reinnervation than muscle physiology experiments. It was also not feasible to perform the donor nerve experiments before the end-to-side repair experiments as it was thought that the repetitive twitch and tetanic stimulation of the donor ulnar nerve that would need to occur proximal to the neurorraphy sites would cause fatigue in the smaller FCR muscle (through the end-to-side pathway) and therefore compromise the function of this muscle and render the end-to-side studies

inaccurate. It was decided that owing to the above problems these experiments would have to be abandoned.

Data for the end-to-side FCU muscle physiology was collected only from the end-to-side group, as the FCR muscles of this group were so degenerate that no electrophysiological tests could be performed on them. However, the numbers of muscles involved did not form a large enough group.

Mass of the Muscles

FCR muscles

The means of the mass and weights of the FCR muscles for all the groups of animals are shown below.

Expt	Mass FCR Mean	Mass FCR Number	Mass FCR Std.dev.	Wt FCR Mean	Wt FCR Number	Wt FCR Std.dev.
E to S	5.17	8	2.50	0.05	8	0.02
DE to S	5.22	12	1.06	0.05	12	0.01
MCONT	10.30	7	2.53	0.10	7	0.02
CONV	5.63	17	1.56	0.06	17	0.01
All Grps	6.18	44	2.53	0.06	44	0.02

Table 52 To show means and standard deviation statistics and number in each group of animals for the mass (g) and weight (N) of the FCR muscles in the experimental groups and normal group.

Breakdown Table of Descriptive Statistics for the mass of FCR (g)

Expt	Mean	Number	Std.dev.	Std.err.	Minimum	Maximum
E to S	5.17	8	2.50	0.89	2.16	8.44
DE to S	5.22	12	1.06	0.30	3.00	7.00
MCONT	10.30	7	2.52	0.96	7.50	14.72
CONV	5.63	17	1.56	0.38	2.97	8.90
All Grps	6.18	44	2.53	0.38	2.16	14.72

Table 53 To show means and standard deviation and error statistics, minimum and maximum values and number in each group of animals for the mass (g) and weight (N) of the FCR muscles in the experimental groups and the normals.

The mass of the FCR and the weight of the FCR were found not to be normally distributed and underwent an F test with the Kruskal-Wallis test to look for differences. Both these variables were found to have values that statistically differed significantly between the groups of animals (p=0.001).

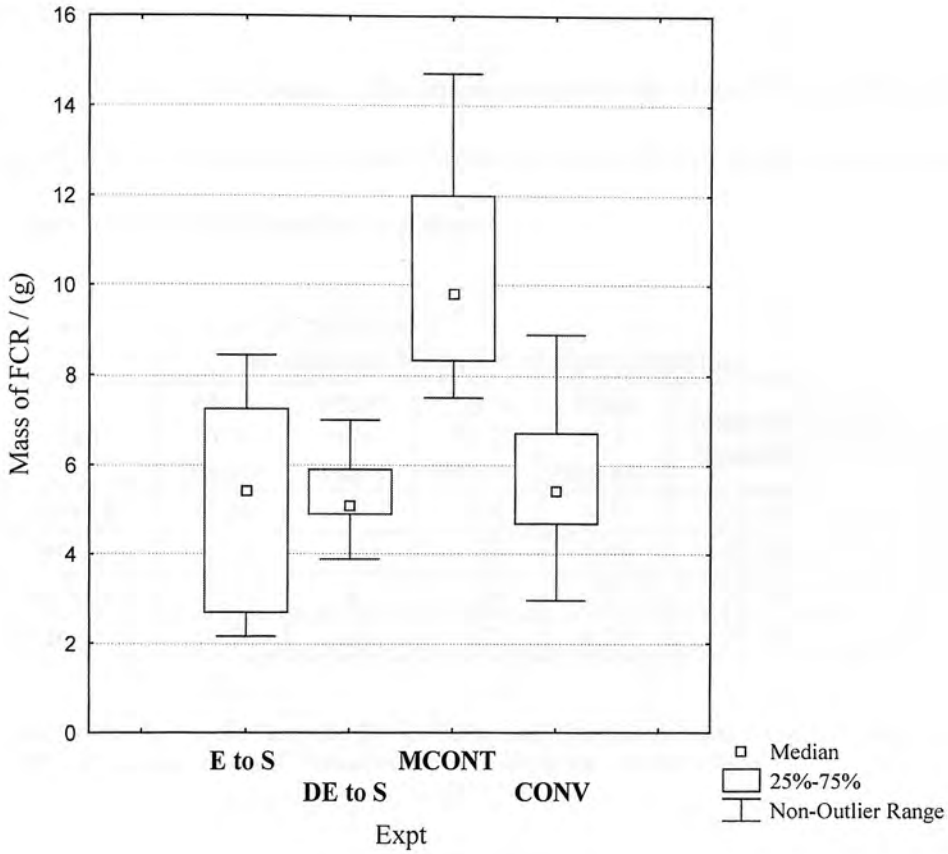
Mann-Whitney U Test (Mass (g) and weight (N) of FCR) Marked tests are significant at p <0.050 By variable: Expt				
	E to S	DE to S	CONV	MCONT
E to S		0.970	0.711	***0.001***
DE to S	0.970		0.744	***0.000***
CONV	0.711	0.744		***0.000***
MCONT	***0.001***	***0.000***	***0.000***	

Table 54 To show where the significant differences in the mass and weight of the FCR muscles between the groups of animals lie.

The mass of the normal FCR muscles are significantly larger than any of the FCR muscles in the experimental groups. This is probably what one might expect as muscles regenerating from neurotmesis and subsequent repair probably never regain

normal contractile properties. The recovery of function of muscle after nerve crush and nerve transection and repair by epineurial suture in rabbit peroneal nerves were investigated by Gutmann in 1942. He found that stimulating the nerve produced contraction of the muscle at 18-20 days after nerve crush and full return of the toe-spreading reflex at 26-28 days. The return of muscle contraction in response to nerve stimulation was slower after nerve transection and suture and the toe-spreading increased only gradually and never returned to normal.

There was a large standard deviation in the weights of the FCR muscles for the end-to-side group compared to those of the other experimental groups. Many of the muscles in the end-to-side group were noticeably wasted and probably consisted of dense fibrous tissue rather than muscle fibres. This could account for the fact that even though these muscles looked a lot smaller to the naked eye their weights were similar to the weights of the muscles of the other groups because they consisted of densely packed fibrous tissue cells. This could also account for the large range in weights of the FCR muscles in the end-to-side group, as some of the muscles actually looked as if they had regenerated well.



Graph 13 To compare the differences in the masses of the FCR muscles between the different groups of animals. There were significant differences between the masses of the muscles in the experimental groups compared to normal.

FCU muscles

Results of the means of the masses in grams (g) of the FCU muscles for the end-to-side and normal groups are shown in the table below along with the number in each group and the standard deviations.

Breakdown Table of Descriptive Statistics

Expt	Mass FCU Means	Mass FCU Number	Mass FCU Std. dev.	Mass FCU Std. err.	Mass FCU Minimum	Mass FCU Maximum
E to S	25.86	5	8.34	3.73	20.40	40.00
DE to S	35.65	12	7.11	2.05	27.00	50.90
UCONT	37.59	8	4.30	1.52	33.20	47.00
All Grps	34.32	25	7.72	1.54	20.40	50.90

Table 55 To show means and standard deviation and error statistics and number in each group of animals for the mass of the FCU muscles in end-to-side groups and normal group.

The mass of the FCU muscle was normally distributed. An F test was performed on this data to look for significant differences between the groups.

Analysis of Variance: Marked effects are significant at $p < .050$

	SS	df	MS	SS	df	MS	F	P
Mass FCU	464.76	2	232.38	964.28	22	43.83	5.302	***0.013***

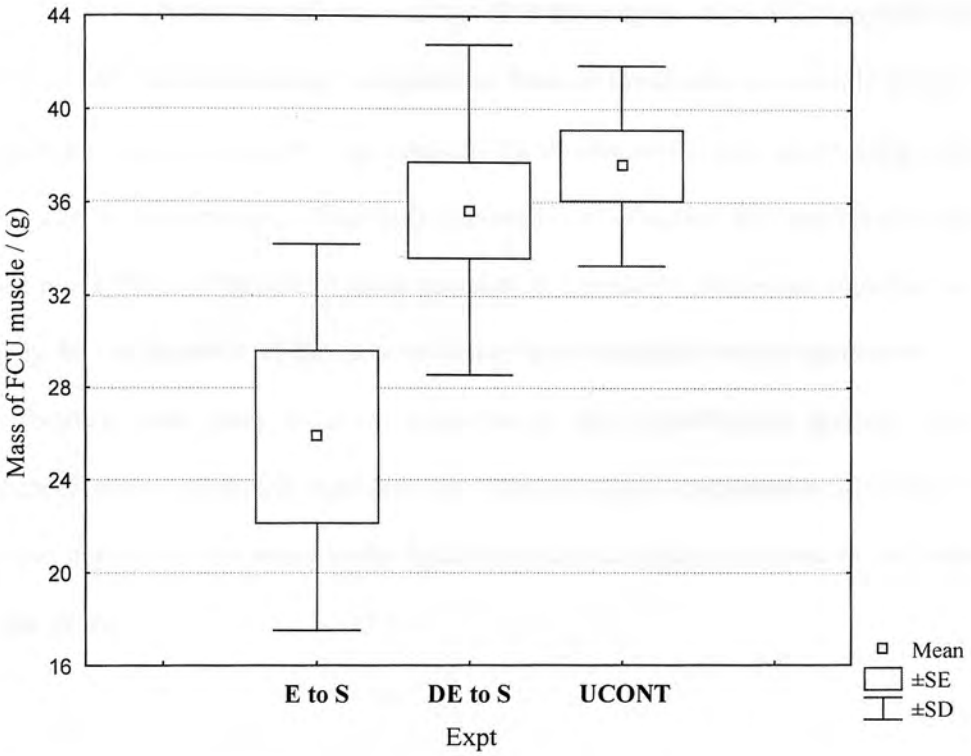
Table 56 To show that there are significant differences in mass of the FCU between the groups of animals (p= 0.013).

Scheffé Test
Variable: **Mass (g) FCU**
Marked differences are significant at $p < .05000$

	{1}	{2}	{3}
E to S {1}		***0.036***	***0.018***
DE to S {2}	***0.036***		0.817
UCONT {3}	***0.018***	0.817	

Table 57 To show the post hoc tests (Scheffé test) on the mass of the FCU. Significant results are shown in bold italic.

The table shows that there are significant differences between the end-to-side group and both the double end-to-side group and the normal ulnar group. The masses of the FCU muscles in the end-to-side group are significantly smaller than in the double end-to-side and normal group. There are no differences between the normal group and the double end-to-side group however.



Graph 14 A box and whisker plot to show the significant differences between the weights of the FCU muscles of the end-to-side group compared to those of normal and the double end-to-side group.

It is perhaps an unusual result to find the masses of the FCU muscles to be less in the end-to-side group compared to those in the double end-to-side group. A double perineurial window was made in the double end-to-side group ulnar nerves for double neurorraphy. Potentially this could cause double the damage created to the nerve fibres of the donor ulnar nerves than the end-to-side group ulnar nerves. It may be the presence of this second lesion that stimulates nerve regeneration more effectively and leads to more rapid nerve fibre growth and quicker muscle reinnervation and muscle regeneration. This more rapid regeneration could lead to a more normal muscle mass in the double end-to-side group compared to the end-to-side group.

Histology - Axon and fibre diameters

The axon and fibre diameters will be discussed together along with the calculated Myelin thicknesses and G-ratios. The results of these histological values from the median nerves will be shown first. Comparisons will be made between the histological values of the normal median nerves, the conventional repair groups and the end-to-side groups. All diameters are in micrometres (μm).

Microscopic view of the nerve sections

On microscopic examination of the slides it was noted that the fibre diameters of the normal nerves were the largest of all the groups. This was followed by the conventional repair groups with the end-to-side group having the smallest fibre diameters. The nerve fibres of the end-to-side groups were extremely difficult to measure especially in the end-to-side group due to greatly diminished numbers and

size of fibres. Unfortunately, a problem occurred during the processing stage of the nerve graft specimens in the araldite. The araldite did not set properly and the specimens had to be re-processed which destroyed the architecture of the nerve fibres on the nerve sections. It was impossible to count axon and fibre diameters from 4 nerve specimens for this group. On examination of the nerve sections of the double end-to-side group it was observed on 6 out of the 14 sections that there were axons growing out with the epineurium. This may mean that axons from the proximal neurorraphy site had grown down the epineurium of the 'bridge' part of the donor nerve towards the distal neurorraphy site. The photographs on the following pages show the nerve sections from each group of animals.

On the end-to-side sections there were also some slides that showed nerve fibres in the epineurial sheaths of the distal part of the median nerve. Collateral or terminal sprouts from the donor nerve may have grown down the epineurial sheath of the attached median nerve to neurotize the end organ directly as well as down the endoneurial tubes (Millesi 2000). However this was a long distance (approximately 10cm in each end-to-side experiment). Some studies on nerve regeneration using conduits have shown that it is not supported well if the conduit is greater than 5cm (Hems & Glasby 1993). Nerve tissue from the 'bridge' area of the double end-to-side group was harvested and nerve fibres were seen in the epineurial layers. The distal nerve tissue that was harvested for processing and examination was approximately 2cm and 3cm from the neurorraphy site (distal neurorraphy site in the double end-to-side group). Nerve axons growing down the epineurial sheath from the neurorraphy site may still be viable at this distance from it but the fate of these axons closer to the end organ is not known histologically. It can be said that

regenerating axons have reached the FCR muscle and formed functional connections, owing to the positive results of the electrophysiological tests and muscle physiology. It cannot be said with any certainty however, by what route the axons arrived at the muscle. This can only be hypothesized from knowledge of nerve regeneration.

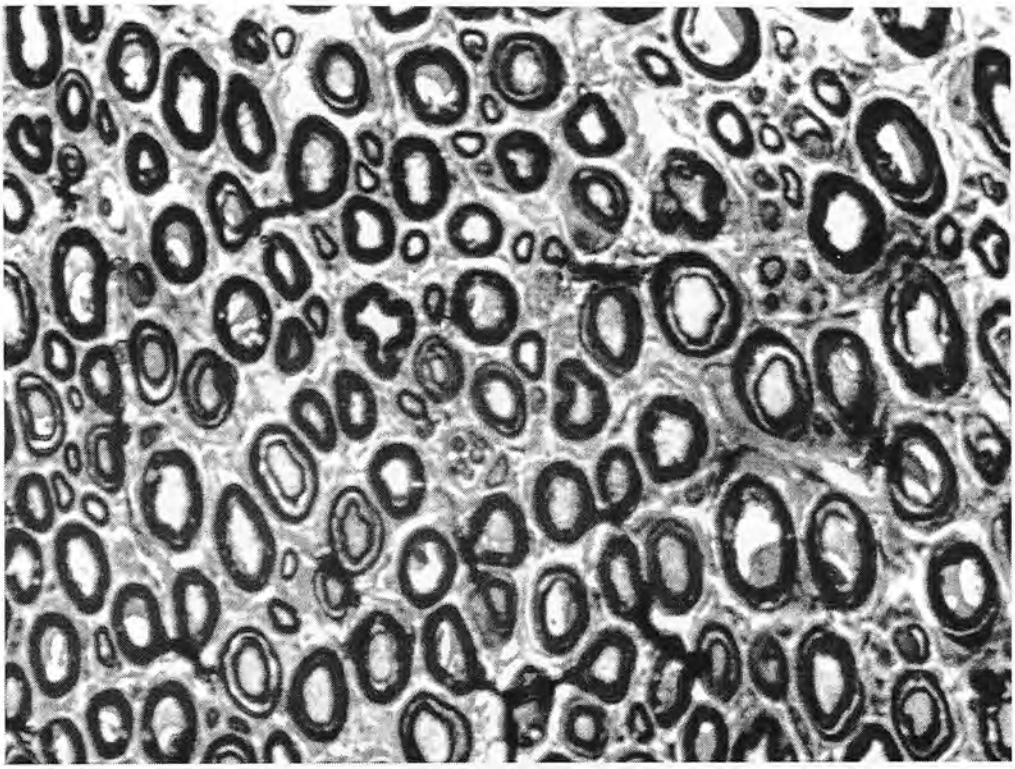


Fig 13 - (x40 objective lens) A cross section through a normal median nerve.

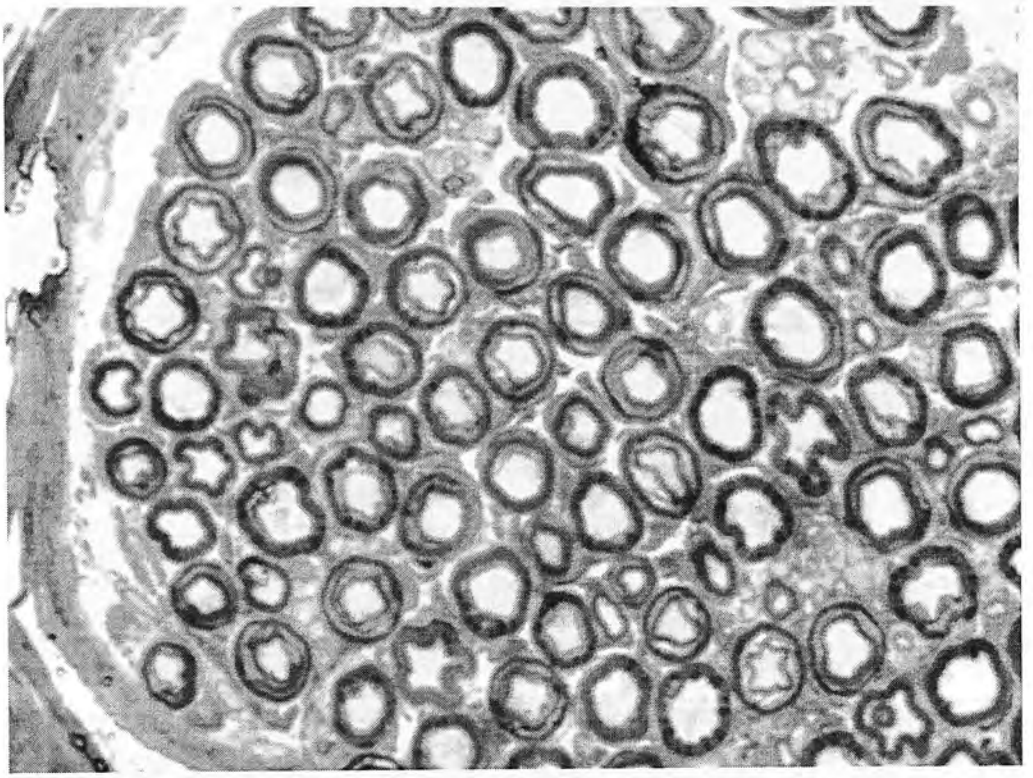


Fig 14 - (x40 objective lens) A cross section through a normal ulnar nerve.

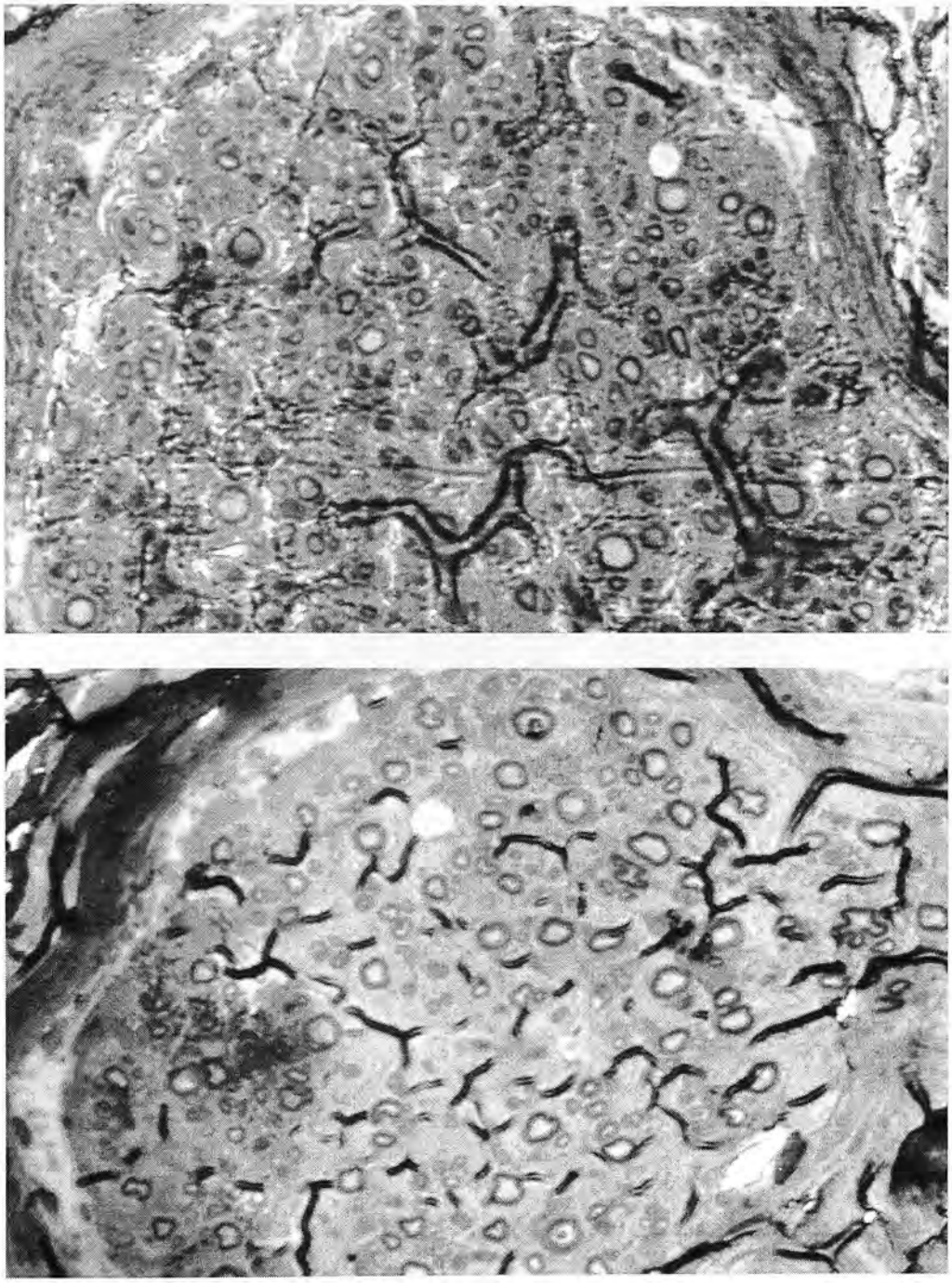


Fig 15 - (x40 objective lens) To show a cross section of one of the double end-to-side median nerve distal to the neurorraphy sites. All nerve fibres are myelinated with some larger in diameter than others.

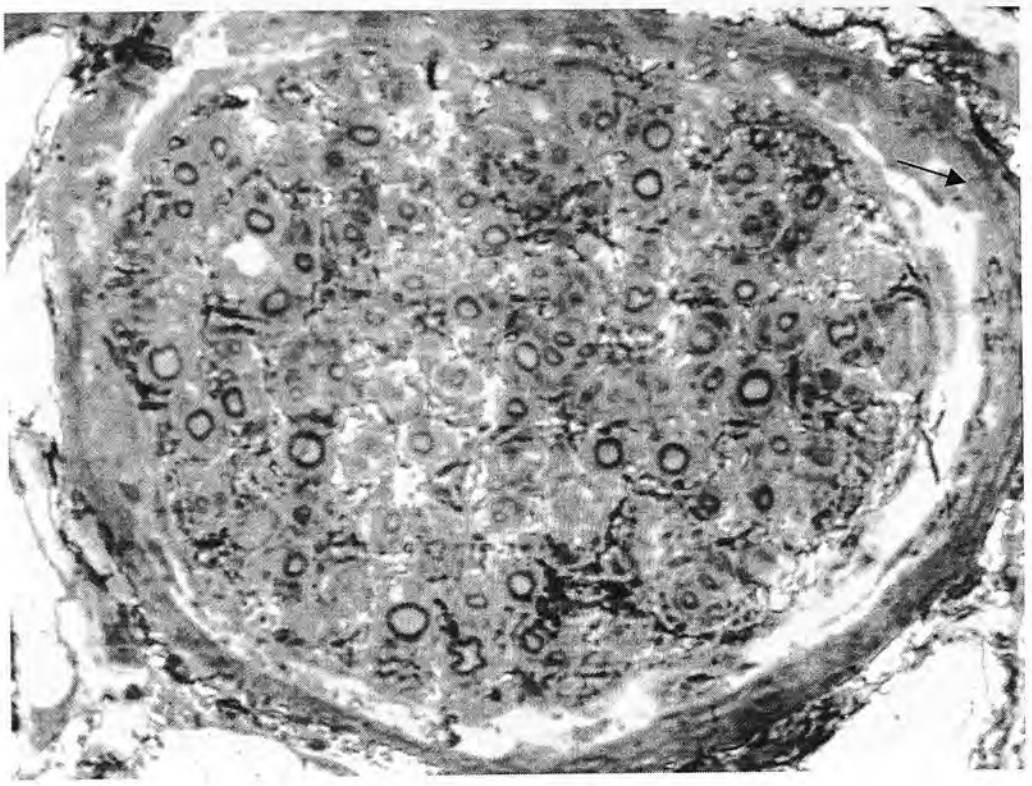


Fig 16 - (x40 objective lens) To show a cross section of one of the double end-to-side median nerve distal to the neurorraphy sites. All nerve fibres are myelinated with some larger in diameter than others. Nerve fibres can be seen the perineurial layer on the right hand side of the picture (arrow).

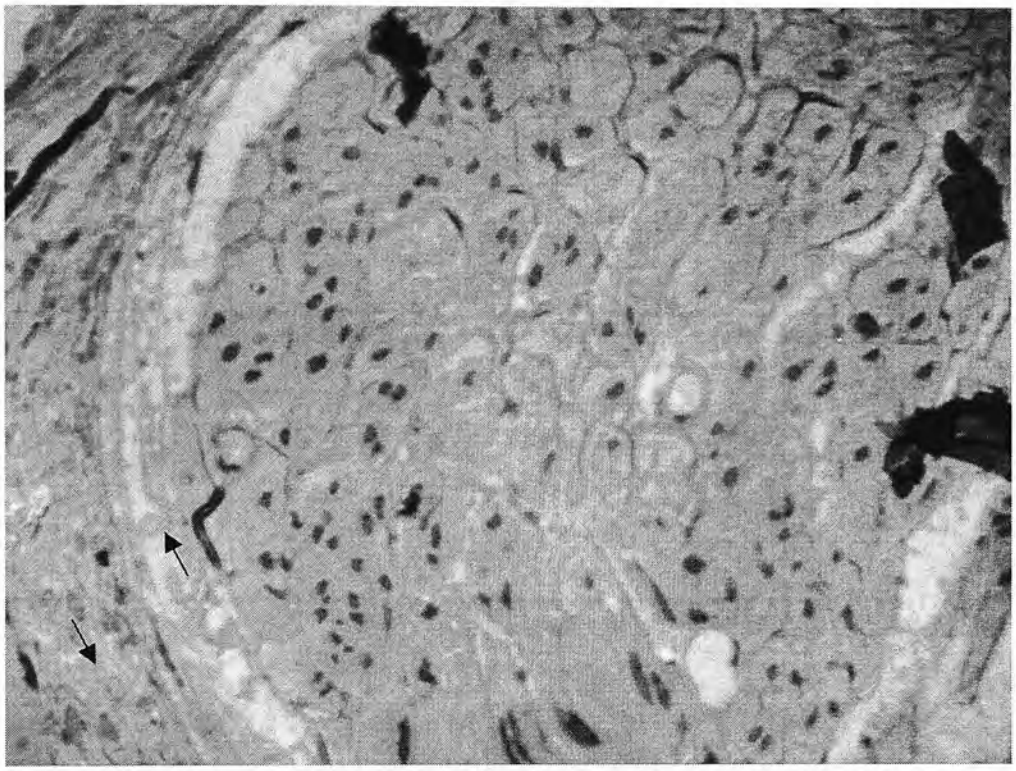


Fig 17 - (x40 objective lens) To show a cross section of a double end-to-side median nerve distal to the neurorraphy sites. All nerve fibres are myelinated with some larger in diameter than others. Nerve fibres can be seen the perineurial layer on the left hand side of the picture (arrows).

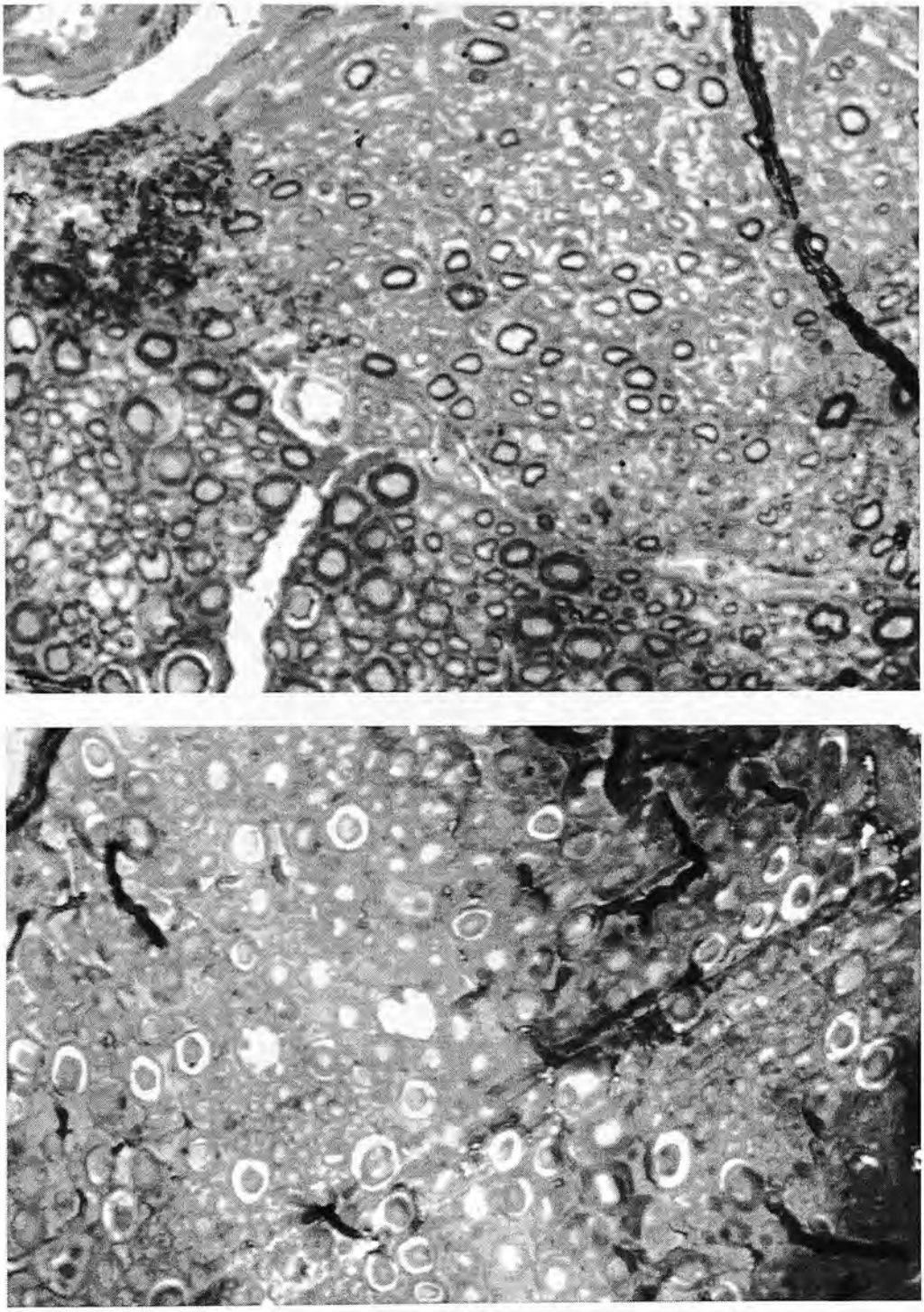


Fig 18 - (x40 objective lens) To show cross sections through two of the double end-to-side donor nerves distal to the neurorraphy sites. All nerve fibres are thinly myelinated with small diameters compared to the normal ulnar nerve sections.

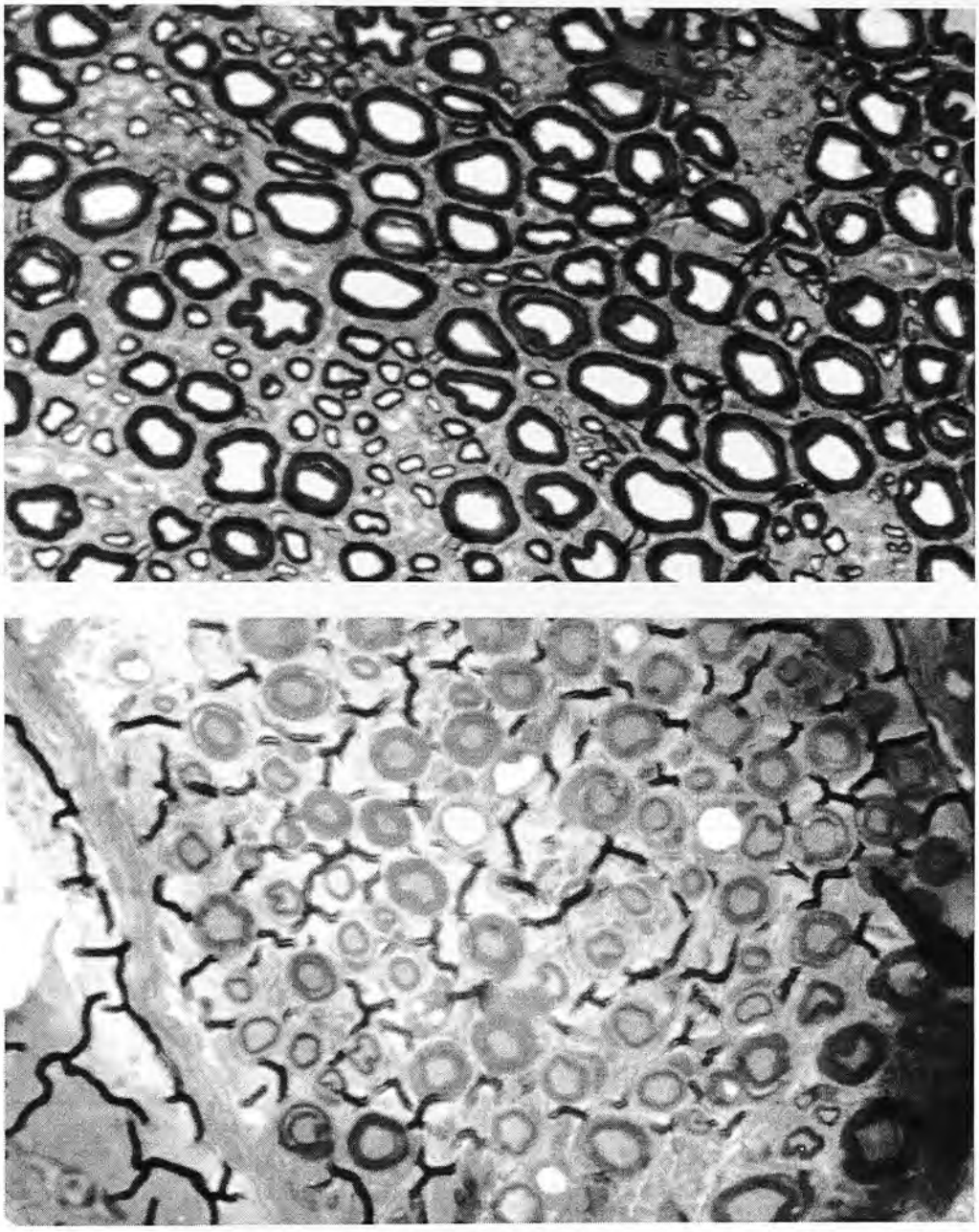


Fig 19 - (x40 objective lens) To show cross sections of two of the more typical double end-to-side donor nerves distal to the neurorraphy sites. All nerve fibres are well myelinated with larger diameters compared to the previous section (Fig 6). The axon and fibre diameters are closer to those of the normal ulnar nerve sections.

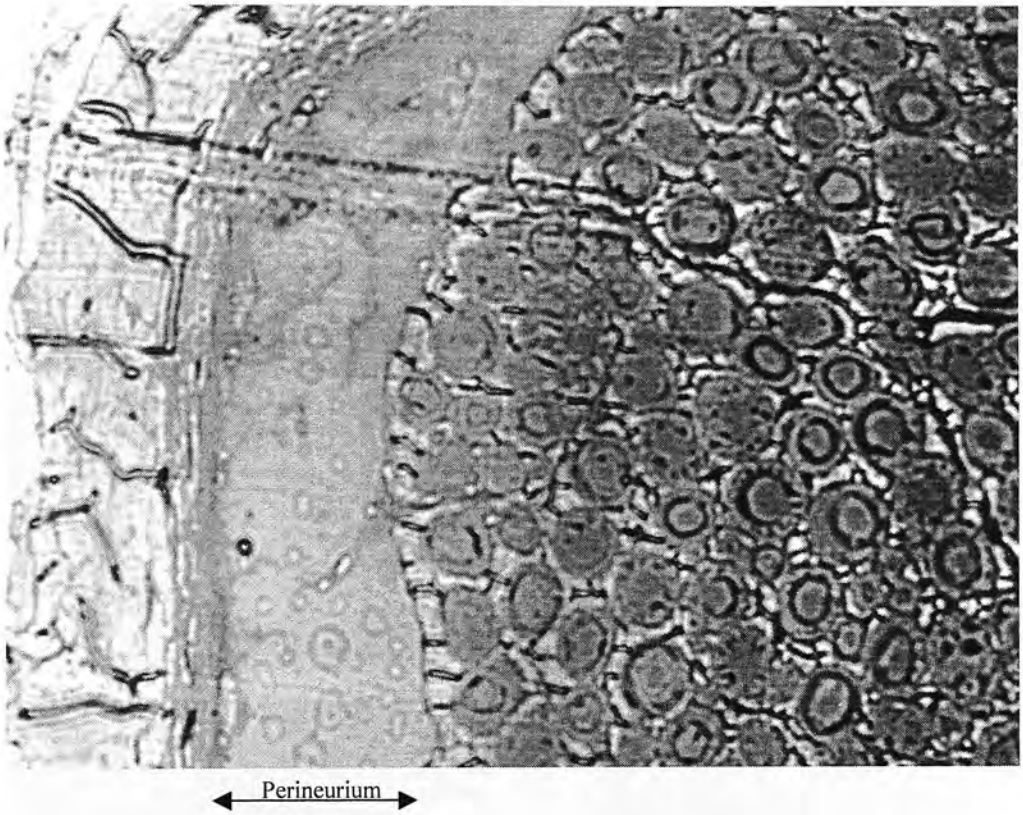


Fig 20 - (x40 objective lens) Cross section through the 'bridge' part of the double end-to-side repair. This shows thinly myleinated axons in the perineurial layers of a fascicle of the donor ulnar nerve.

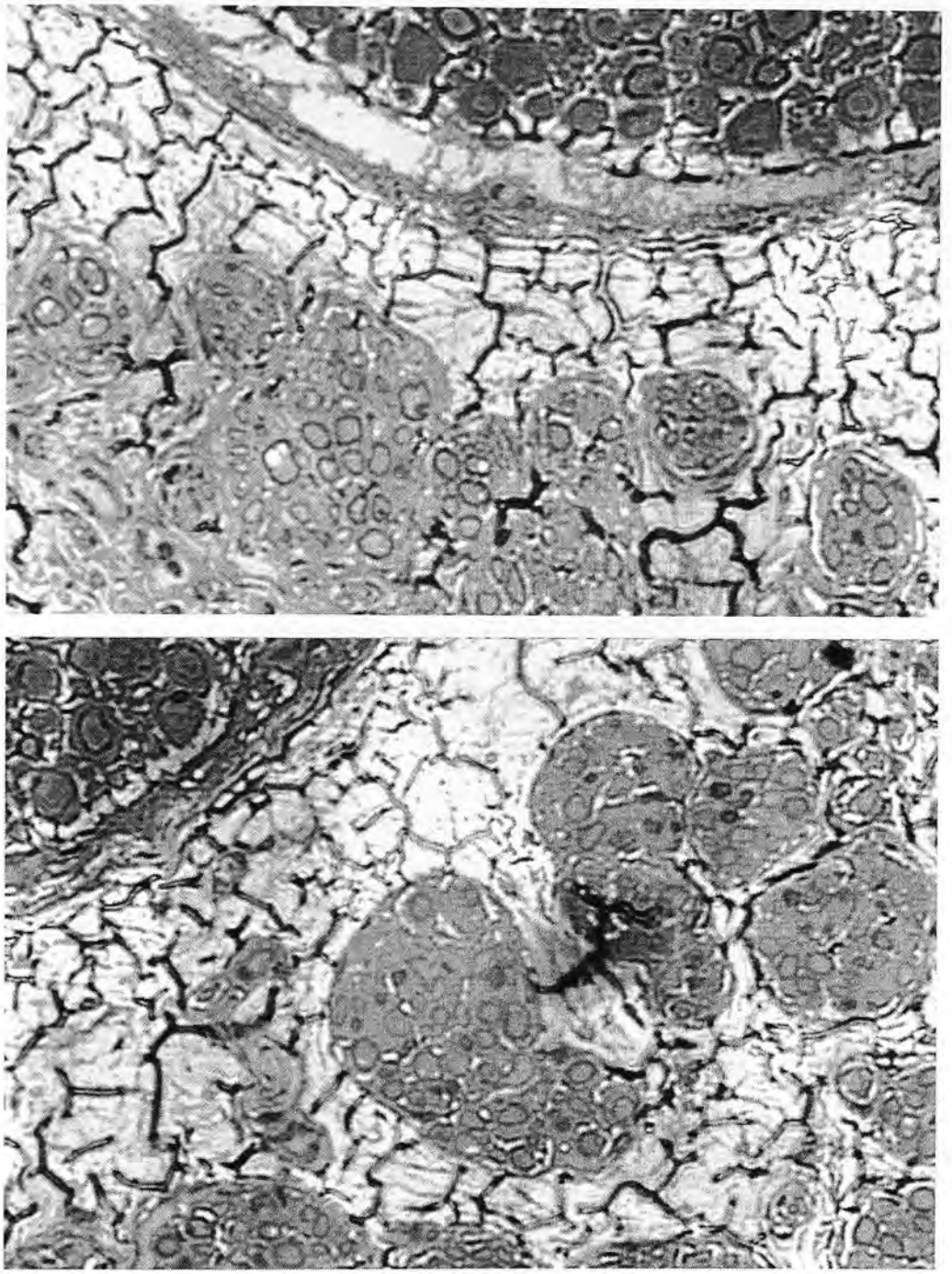


Fig 21 - (x40 objective lens) Cross sections through the 'bridge' part of the double end-to-side repair. Discrete fascicles can be seen in the eipneurium containing thinly myelinated axons. A fascicle of the donor ulnar nerve of the bridge can be see at the top of the top picture and in the top left corner of the bottom picture.

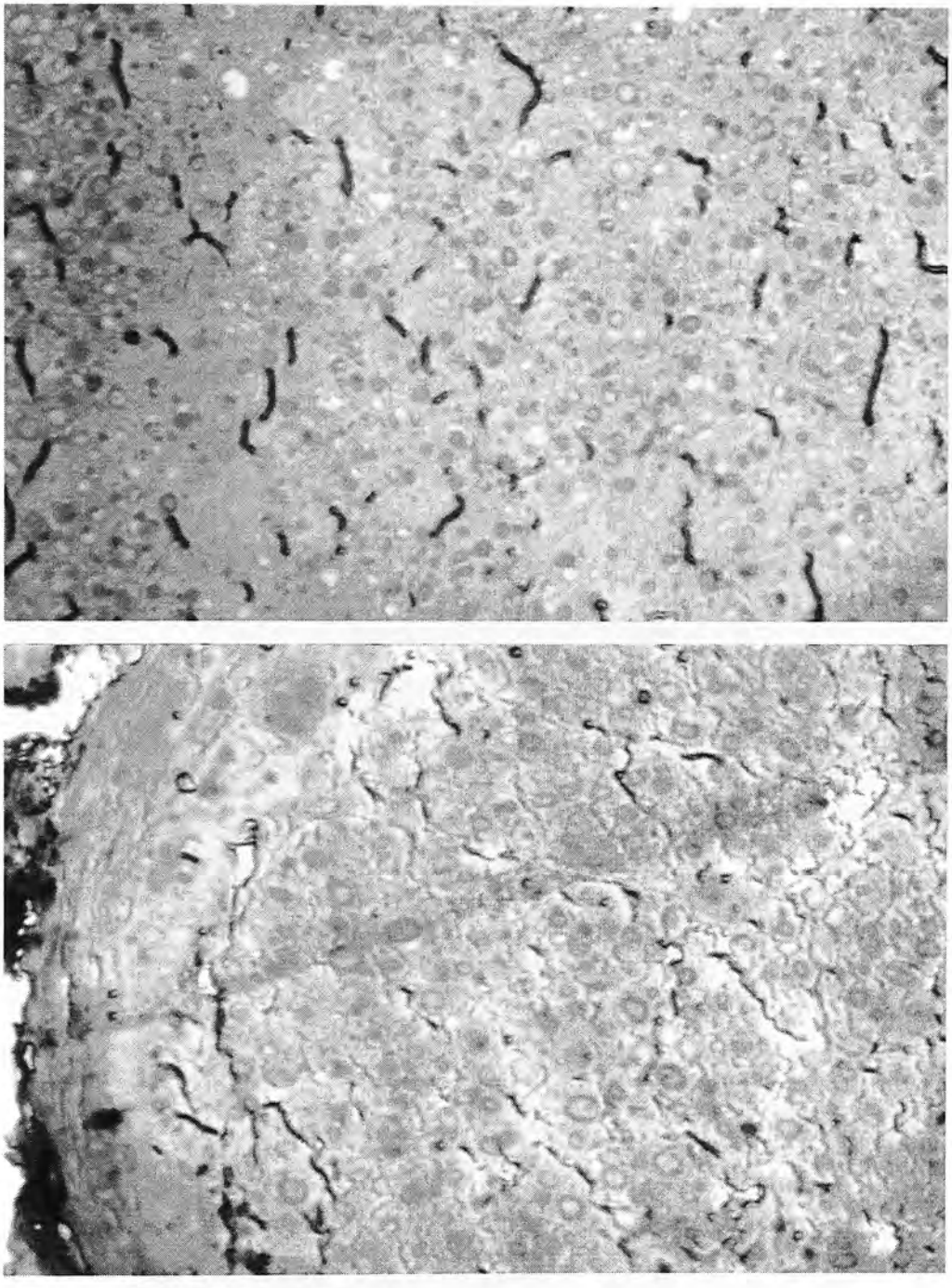


Fig 22 - (x40 objective lens) Cross sections through the attached recipient distal median nerve stump of the end-to-side repair. Thinly myelinated axons of small diameter can be seen in the fascicles of the nerve trunk surrounded by fibrous tissue.

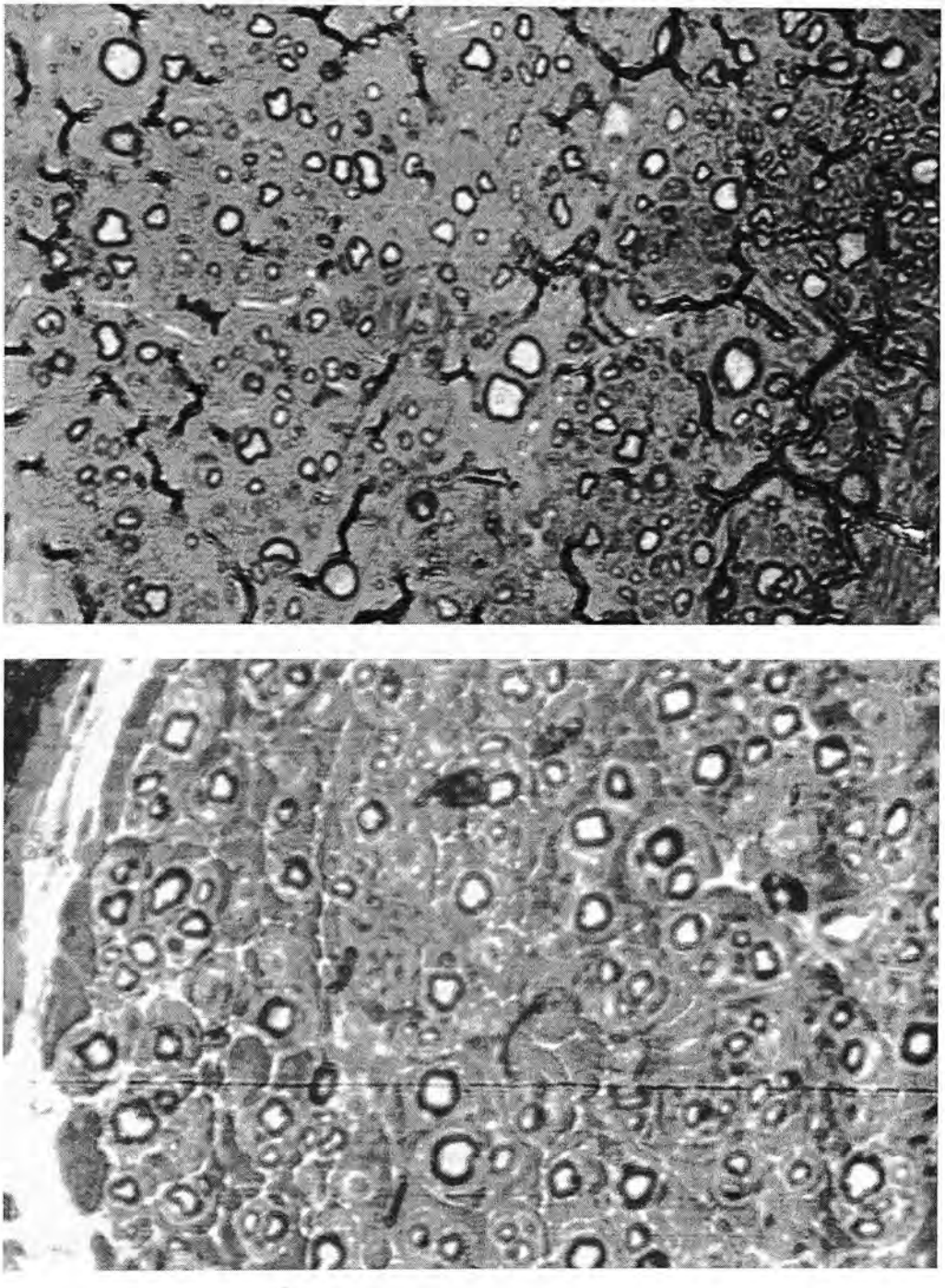


Figure 23 - (x40 objective lens) Cross sections through the recipient distal median nerve of the end-to-side repair. Axons are well myelinated with a wide range of diameters.

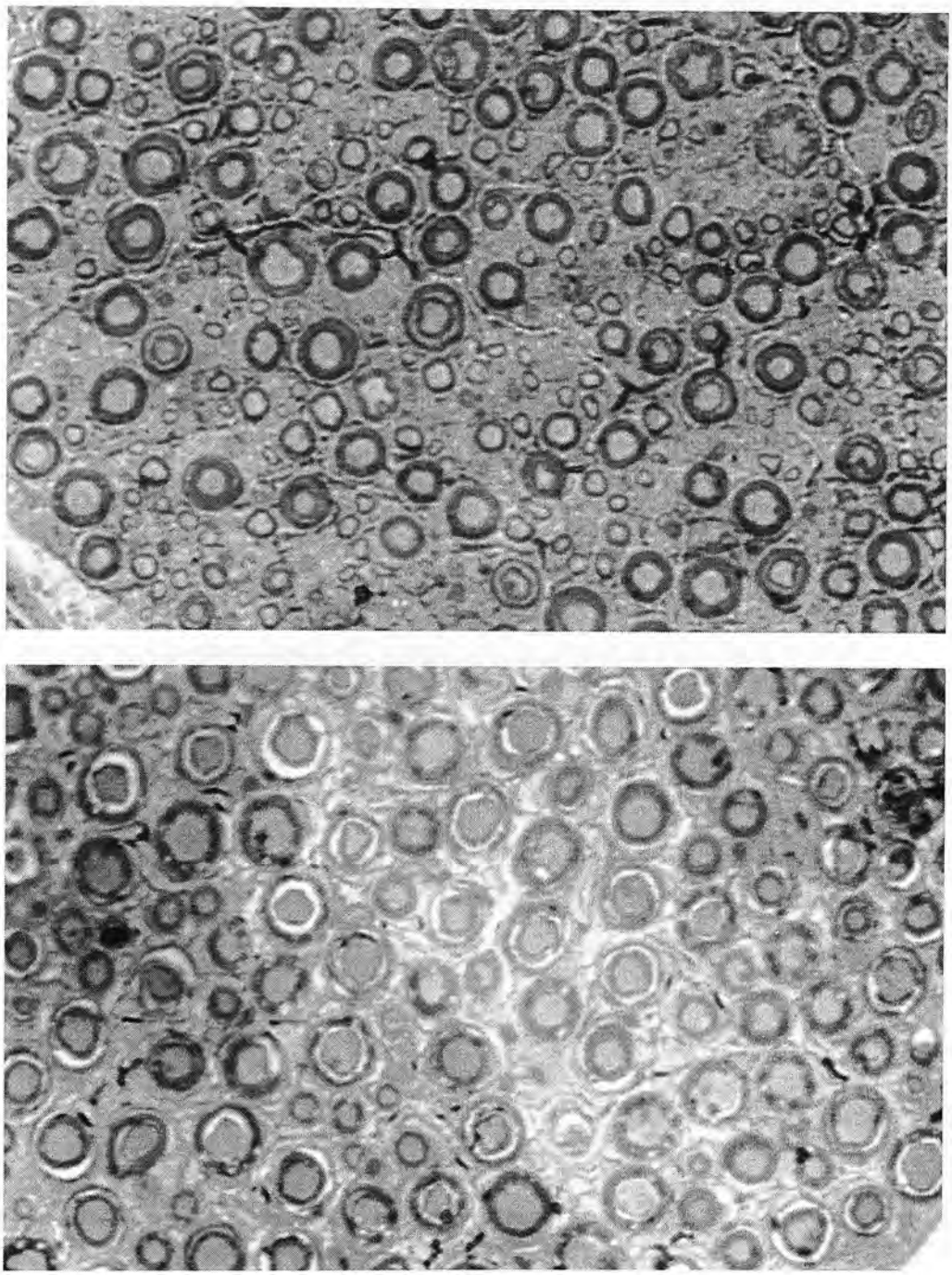


Fig 24 - (x40 objective lens) Cross section through a donor ulnar nerve of the end-to-side repair. Axons vary in diameter and are smaller than normal ulnar nerve axons. All axons are well myelinated.

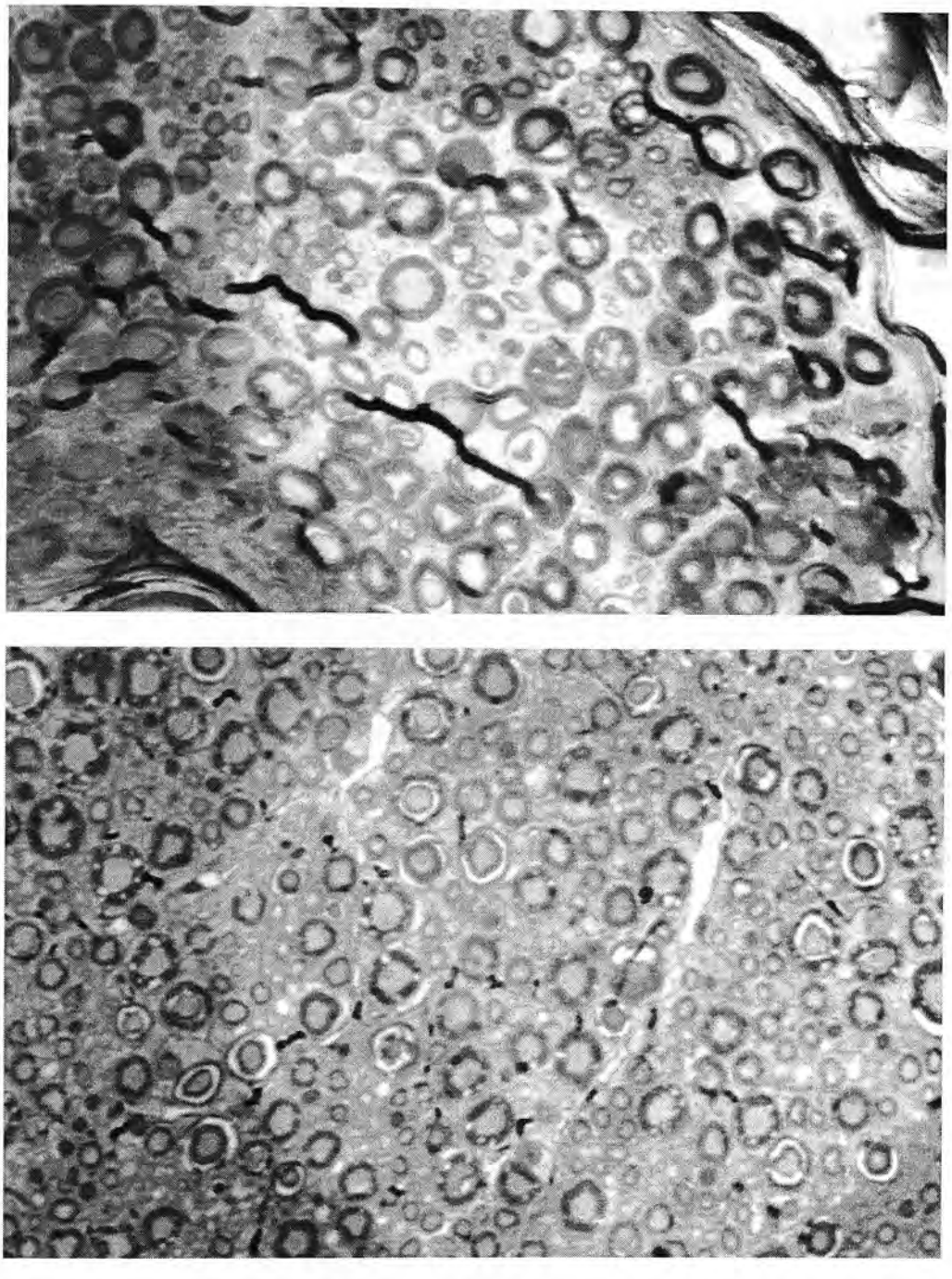


Fig 25 - (x40 objective lens) Cross section through a donor ulnar nerve of the end-to-side repair. Axons vary in diameter and are smaller than normal ulnar nerve axons. All axons are well myelinated.

The microscopic pictures of the recipient attached median nerves of the end-to-side group distal to the neurorraphy sites show a wide variation in the diameter in the nerve fibres between different nerves. This is also true for the double end-to-side group but not to the same degree. Fibres of these groups from the pictures look larger in diameter generally than those of the end-to-side group. The pictures shown here represent the smallest fibres and the largest fibres among the repaired and donor nerves found for each group. There are also a lot more fibres in the fascicles of the double end-to-side group compared to the end-to-side group. Absolute fibre counts may have been useful here to establish whether this observation was correct.

The pictures of the distal sections of the donor ulnar nerve also show large variation in the diameters of the axons for both end-to-side groups. The largest and smallest nerve fibres that could be found among the repaired nerves for each group are shown. In general, the nerve fibres of the double end-to-side group are smaller in appearance on the pictures than the end-to-side group.

Median nerves

The table below shows the mean values and standard deviations of the axon and fibre diameters with calculated myelin sheath thicknesses and G-ratios for all the groups where the median nerve was under test.

Breakdown Table of Descriptive Statistics

Expt	AxonM Mean	AxonM Number	AxonM Std. dev.	FibreM Mean	FibreM Number	FibreM Std. dev.
E to S	2.81	11	0.37	5.21	11	0.73
DE to S	3.40	12	0.53	5.83	12	1.00
MCONT	8.99	5	0.55	16.72	5	0.64
CONV	4.43	11	0.50	8.17	11	0.77
All Grps	4.24	39	2.00	7.71	39	3.78

Table 58

Breakdown Table of Descriptive Statistics

Expt	MyelinM Mean	MyelinM Number	MyelinM Std. dev.	G-ratioM Mean	G-ratioM Number	G-ratioM Std. dev.
E to S	1.20	11	0.20	0.54	11	0.02
DE to S	1.21	12	0.39	0.59	12	0.09
MCONT	3.87	5	0.27	0.53	5	0.02
CONV	1.87	11	0.25	0.54	11	0.04
All Grps	1.73	39	0.92	0.55	39	0.06

Table 59 Tables 58 and 59 show the mean values and standard deviations of these values for the histological measurements of the repaired and normal median nerves.

Results of the conventional repairs compared with the end-to-side groups and the normal group

The individual results for the wrap, neurotmesis and graft groups of animals were compared to each other using the statistical tests used before and there were found to be no significant differences among them for any of the histological values. These groups were combined to become the 'conventional repair' group.

The histological variables were tested for normality after rejecting outliers and found not to be normally distributed. An F test for non-parametrically distributed data (Kruskal-Wallis) was applied to this data to look for variation in the

mean values of the histological variables between the groups of animals. It was found that there were significant differences in the mean values between the groups for the axon and fibre diameter and myelin sheath thickness but not for the G-ratio as shown below:

Kruskal-Wallis ANOVA by Ranks; **G-RatioM**
Independent (grouping) variable: **Expt**
Kruskal-Wallis test: H =6.982319, p =0.0725

	Valid Number	**Sum of Ranks**
E to S	11	189.00
DE to S	12	325.00
MCONT	5	72.00
CONV	11	194.00

Table 60

Kruskal-Wallis ANOVA by Ranks; **AxonM**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =29.36031, **p =0.0000**

	Valid Number	**Sum of Ranks**
E to S	11	90.00
DE to S	12	198.00
MCONT	5	185.00
CONV	11	307.00

Kruskal-Wallis ANOVA by Ranks; **FibreM**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =28.74662, **p =0.0000**

	Valid Number	**Sum of Ranks**
E to S	11	105.00
DE to S	12	178.00
MCONT	5	185.00
CONV	11	312.00

Tables 61 and 62

Kruskal-Wallis ANOVA by Ranks; **MyelinM**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =26.23566, **p =0.0000**

	Valid Number	**Sum of Ranks**
E to S	11	127.00
DE to S	12	162.00
MCONT	5	185.00
CONV	11	306.00

Table 63. Tables 60, 61, 62 and 63 show the f test (Kruskal-Wallis) results for the G-ratio of the median nerves in the groups shown. p=0.0725, which shows there were no significant differences between the groups.

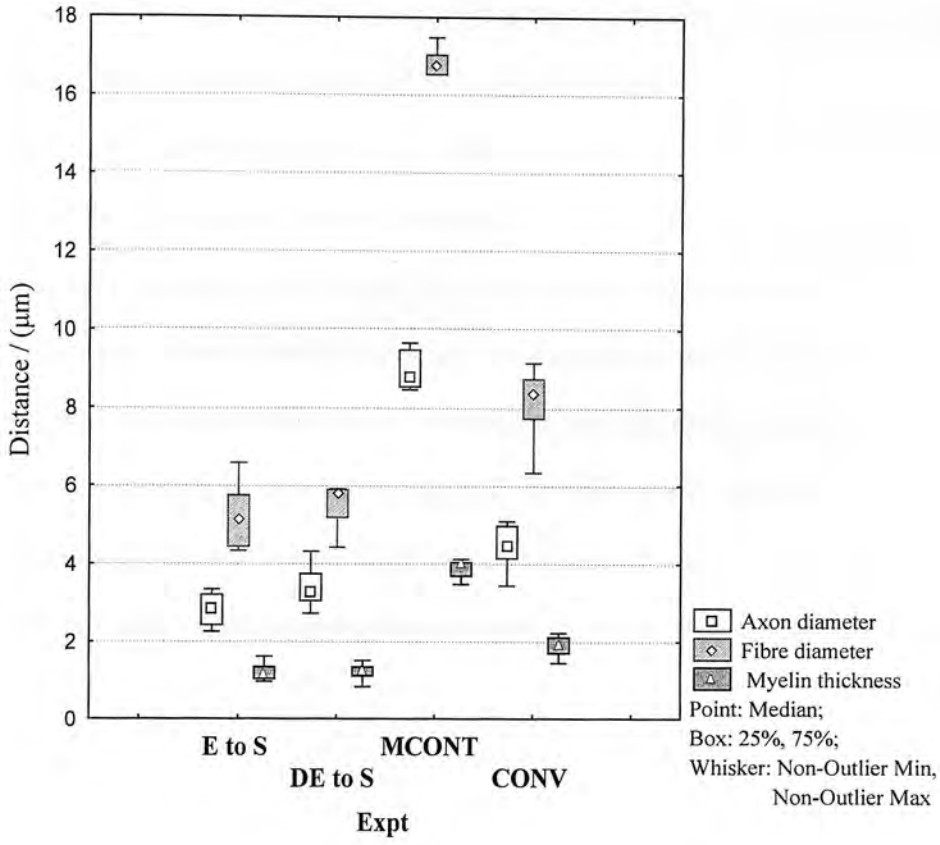
Mann-Whitney U Test (axon diameter of median nerve sections) Marked tests are significant at p <0.050 By variable: Expt				
	E to S	DE to S	CONV	MCONT
E to S		***0.009***	***0.000***	***0.000***
DE to S	***0.009***		***0.000***	***0.000***
CONV	***0.000***	***0.000***		***0.000***
MCONT	***0.000***	***0.000***	***0.000***	

Mann-Whitney U Test (fibre diameter of median nerve sections) Marked tests are significant at p <0.050 By variable: Expt				
	E to S	DE to S	CONV	MCONT
E to S		0.091	***0.000***	***0.000***
DE to S	0.091		***0.000***	***0.000***
CONV	***0.000***	***0.000***		***0.000***
MCONT	***0.000***	***0.000***	***0.000***	

Mann-Whitney U Test (myelin thickness of median nerve sections) Marked tests are significant at p <0.050 By variable: Expt				
	E to S	DE to S	CONV	MCONT
E to S		0.651	***0.000***	***0.000***
DE to S	0.651		***0.000***	***0.000***
CONV	***0.000***	***0.000***		***0.000***
MCONT	***0.000***	***0.000***	***0.000***	

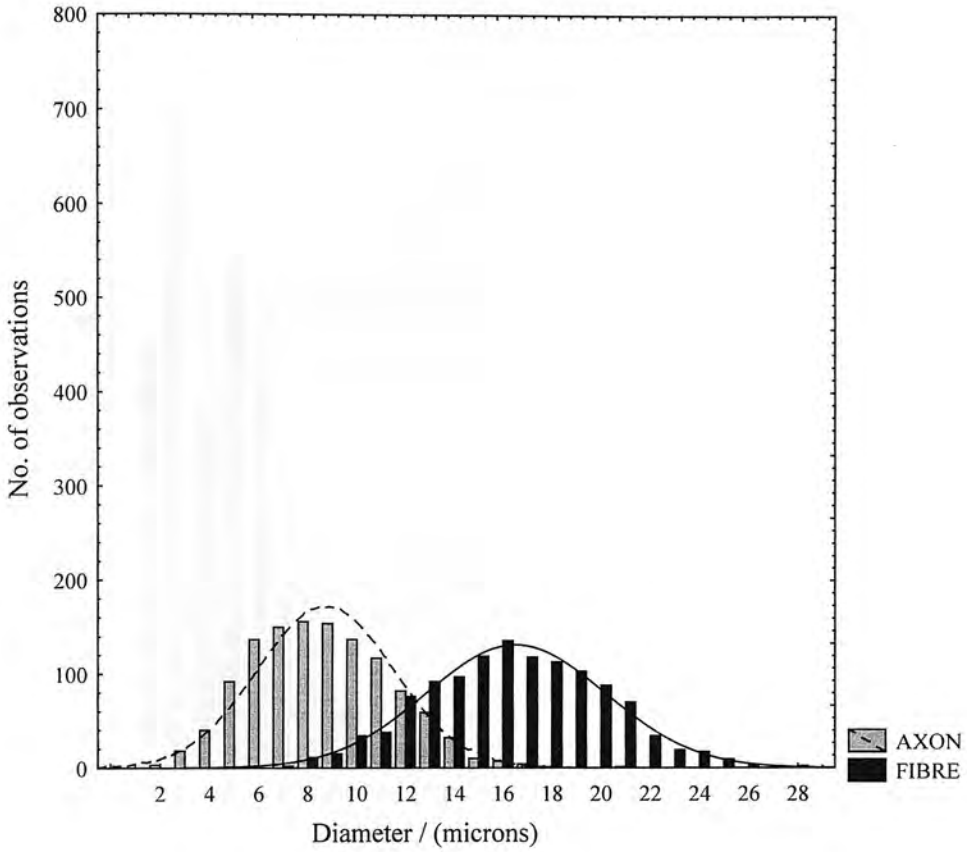
Table 64 To show where the significant differences in the axon and fibre diameter and myelin thicknesses of the median nerve sections of the repaired nerve groups and normal median group lay.

The G-ratio not being significant means that the change in the axon and fibre diameters and the myelin sheath have reduced in the experimental groups compared to the normal groups in proportion. This indicates the regenerated nerve fibres are mature. The greatest axon, fibre and myelin sheath diameters are in the normal group followed by the conventional repair group, then double end-to-side and lastly the end-to-side group. The box plot below shows this relationship well.

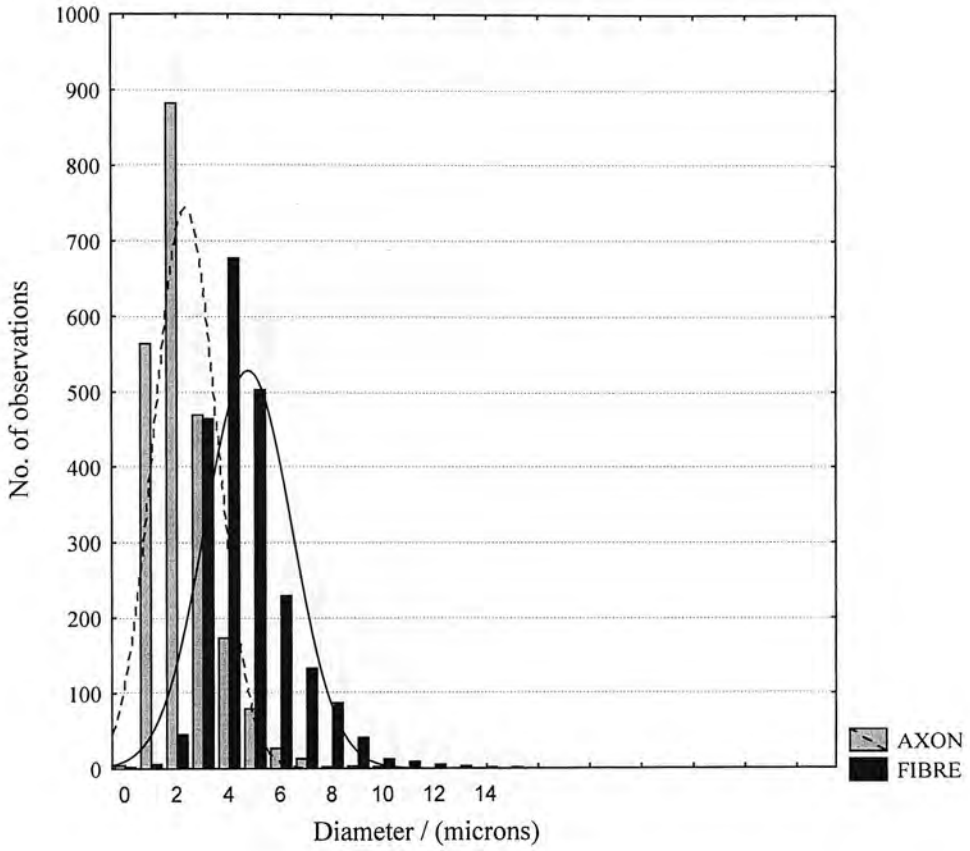


Graph 15 Box and whisker plot to show the differences in the axon and fibre diameters and myelin sheath thicknesses in micrometres (µm) of the median nerves distal to the site of nerve repair between the experimental groups and normal.

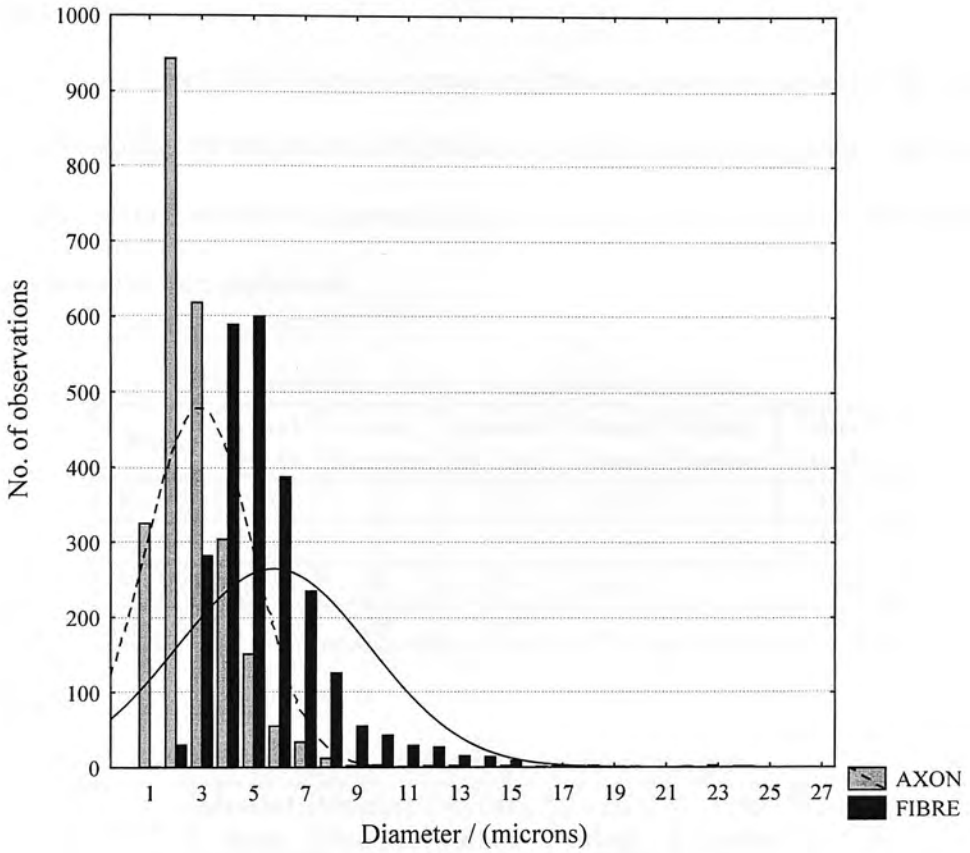
The histograms below show the distribution of the axon and fibre diameters for the normal median nerves and the distal segments of the median nerves of the end-to-side and double end-to-side groups. There is an obvious shift to the left for both these experimental groups compared to normal. The axon and fibre diameters have both decreased. The G-ratio for the experimental groups shows no differences in the mean values compared to normal median nerves, therefore the myelin sheath thickness has become thinner in proportion to the decreased axon diameters. This observation suggests that the regenerated nerve fibres are mature, which does not correspond with the findings of the 'jitter' experiments for the FCR muscles of the median nerves supplied by the repaired median nerves from the end-to-side groups.



Graph 16 Histogram to show a normal model distribution of axon and fibre diameters for a normal median nerve



Graph 17 Histogram to show a normal model distribution of axon and fibre diameters for the distal segment of the median nerve of the end-to-side group.



Graph 18 Histogram to show a normal model distribution of axon and fibre diameters for the distal segment of the median nerve of the double end-to-side group.

Ulnar nerves

The tables below shows the mean and standard deviation values for the ulnar nerve histology for the end-to-side groups and normal ulnar nerve group. The ulnar nerves in the end-to-side groups are the donor nerves to which end-to-side neurorraphys were performed.

Breakdown Table of Descriptive Statistics

Expt	AxonU Mean	AxonU Number	AxonU Std. dev.	FibreU Mean	FibreU Number	FibreU Std. dev.
E to S	6.86	11	1.28	11.09	11	1.82
DE to S	6.26	10	1.39	11.51	10	2.51
UCONT	8.78	8	1.34	14.60	7	2.08
All Grps	7.18	29	1.65	12.12	28	2.54

Table 65

Breakdown Table of Descriptive Statistics

Expt	MyelinU Mean	MyelinU Number	MyelinU Std. dev.	G-ratioU Mean	G-ratioU Number	G-ratioU Std. dev.
E to S	2.11	11	0.34	0.62	11	0.03
DE to S	2.62	10	0.77	0.55	10	0.06
UCONT	2.90	7	0.33	0.60	7	0.02
All Grps	2.49	28	0.61	0.59	28	0.05

Table 66. Table 65 and 66 show the means, standard deviations and number in each group of animals for the histological values of the ulnar nerves

All histological values were found to be normally distributed except the G-ratio. The results of the f-test for the normal and non-normal data are shown below. The Kruskal-Wallis test was performed on the G-ratio values for the groups and has shown that there are significant differences between the groups:

Kruskal-Wallis ANOVA by Ranks; **G-ratioU**
Independent (grouping) variable: Expt
Kruskal-Wallis test: H =10.70446, p =***0.005***

	Valid	**Sum of**
E to S	11	211.00
DE to S	10	78.00
UCONT	7	117.00

Table 67 To show the results of the Kruskal-Wallis F test on the non-parametrically distributed data. There are significant differences, p=0.005.

Analysis of Variance: Marked effects are significant at p < .05000

	SS	**df**	**MS**	**SS**	**df**	**MS**	**F**	**p**
AxonU	30.11	2	15.05	46.45	26	1.79	8.427	***0.002***
FibreU	58.11	2	29.06	115.86	25	4.63	6.270	***0.006***
MyelinU	2.91	2	1.45	7.25	25	0.29	5.016	***0.015***

Table 68 To show the results of the one-way ANOVA F test for the normally distributed histological data. Significant variation in the mean values for the 3 variables listed in the table above exists.

The F tests above show that there are significant differences for all the histological data between the groups. The data then underwent further tests to find between which groups these differences lay. The normally distributed data underwent the Scheffè test and the non-normal data the Mann Witney-U test.

Scheffé Test
Variable: **AxonU**
Marked differences are significant at
p <0.050

	{1}	{2}	{3}
E to S {1}		0.592	***0.017***
DE to S {2}	0.591		***0.002***
UCONT {3}	***0.017***	***0.002***	

Scheffé Test
Variable: **FibreU**
Marked differences are significant at
p <0 .05000

	{1}	{2}	{3}
E to S {1}		0.907	***0.009***
DE to S {2}	0.907		***0.026***
UCONT {3}	***0.009***	***0.026***	

Scheffé Test
Variable: **MyelinU**
Marked differences are significant at
p < .05000

	{1}	{2}	{3}
E to S {1}		0.115	***0.021***
DE to S {2}	0.115		0.593
UCONT {3}	***0.021***	0.593	

Tables 69, 70 and 71 To show where the differences for each group of animals lay for each histological parameter. Table 1 – axon diameter, table 2 – fibre diameter and table 3 - myelin thickness.

The above tables show there were differences between the normal group and the end-to-side groups for the axon and fibre diameters but for the myelin thickness the differences between the controls and the end-to-side group only. There were no significant differences between the end-to-side groups for any of these histological data.

Mann-Whitney U Test (G-ratio of ulnar nerves) Marked tests are significant at p <0.050 By variable: Expt			
	E to S	DE to S	UCONT
E to S		***0.002***	0.480
DE to S	***0.002***		***0.019***
UCONT	0.480	***0.019***	

Table 72 To show where the significant differences in the G-ratio of the ulnar nerves between the groups of animals lay.

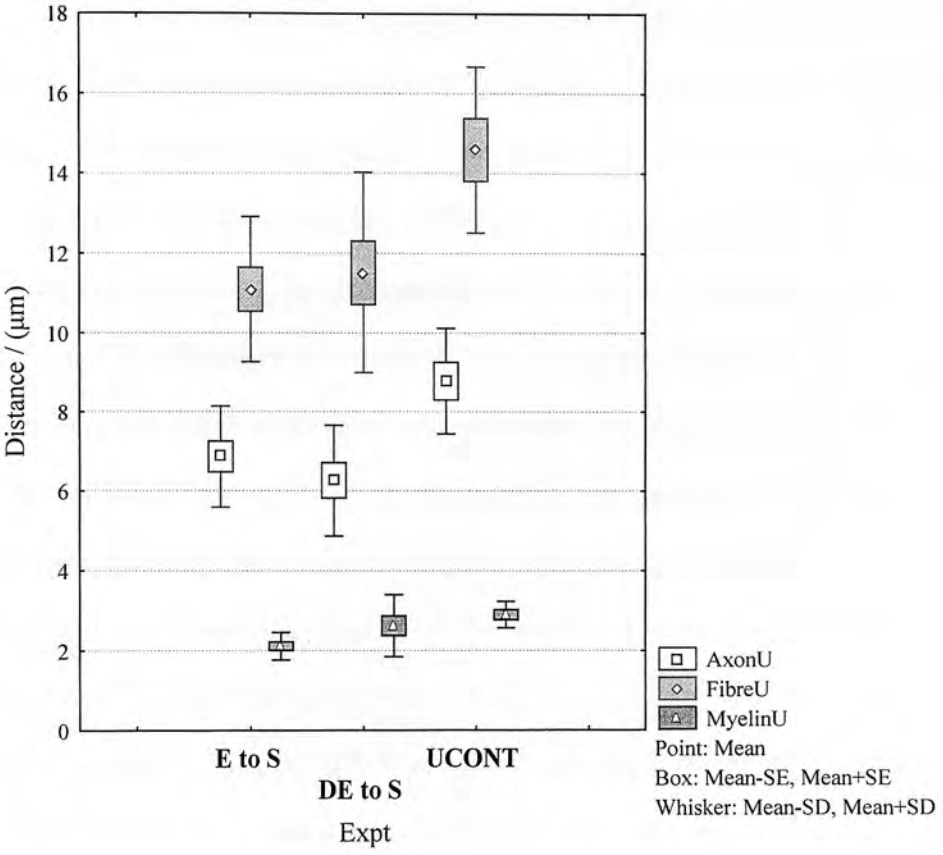
The Mann Witney-U tests on the non-parametrically distributed G-ratio show that the mean values are significantly different between the end-to-side groups and between the double end-to-side group and normal. There were no differences between the end-to-side group and normal. The box and whisker plots show these findings well.

In both end-to-side groups the axon and fibre diameters have decreased significantly compared to normal. The mean axon diameter for the double end-to-side group is smaller than that for the end-to-side group but is not statistically significant different.

These results suggest that damage to the axons of the ulnar nerve may have occurred during epineurotomy. While the results suggest that regeneration of axons is complete in the end-to-side group (normal G-ratio and jitter), this is not the case for the double end-to-side groups where, as for the median nerves, the jitter and G-ratio values measured contradict.

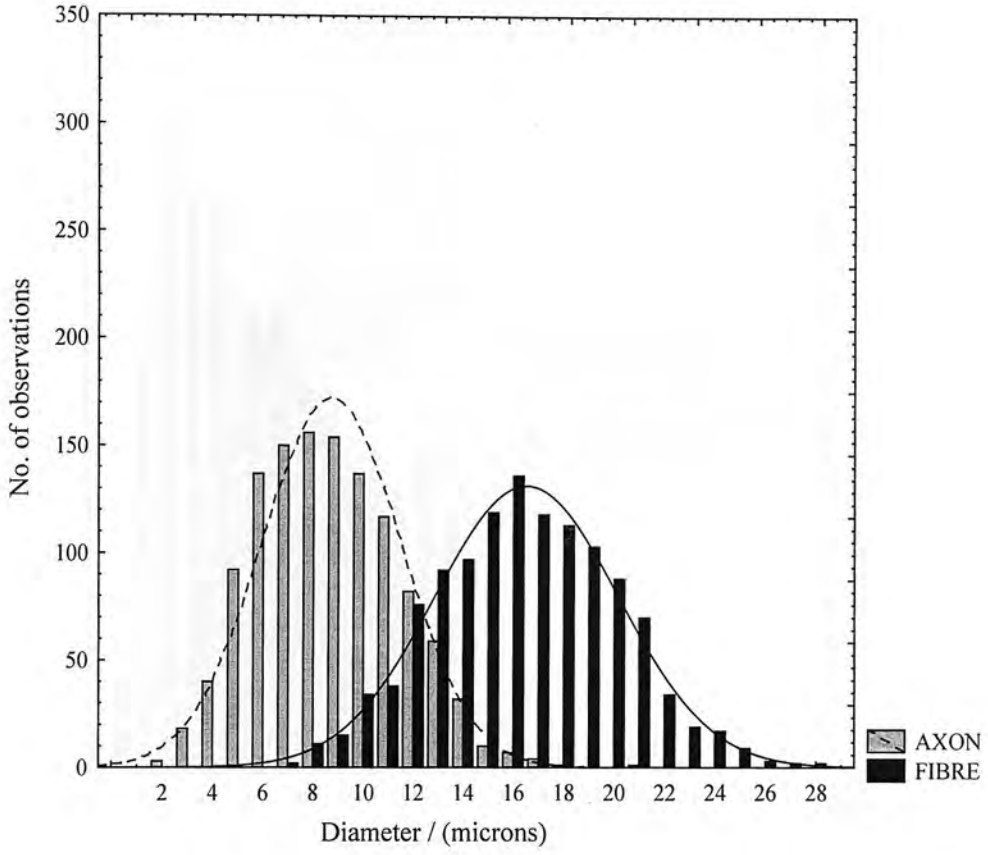
The results of the mean jitter values and those of the G ratios for the end-to-side group contradict one another to some extent. The mean jitter values are significantly higher in this group compared to the other experimental groups and

normal. This suggests that nerve regeneration is not as complete in this group compared with the other groups as neuromuscular transmission is not as stable. Jitter assesses transmission of the nerve electrical impulse from the motor end plate to the muscle fibres. This is physiologically a different process from 'electrical' transmission down a nerve trunk. It is therefore difficult to say that because the regenerated axons from the end-to-side repair group are probably mature because the myelin sheath thickness is of a size that is proportional to the axon diameter that transmission from the motor end plate distally to the muscle fibres is equally as mature. We do know that this stability may never completely return to normal in a repaired nerve but may in smaller animals (Lenihan 2000). Jitter measures this stability and we do know that this value seems to relate to the maturity of nerve regeneration, a lower value suggesting that nerve regeneration is nearer to completion (Lenihan 2000). The high values for jitter with G-ratios comparable to those for normal nerve axons in the end-to-side group, suggests that nerve regeneration is complete up to the motor end plate but is still continuing past it towards the nerve fibres. This is reflected in the lower, although not statistically significant, values for the muscle twitch and tetanic tension values.

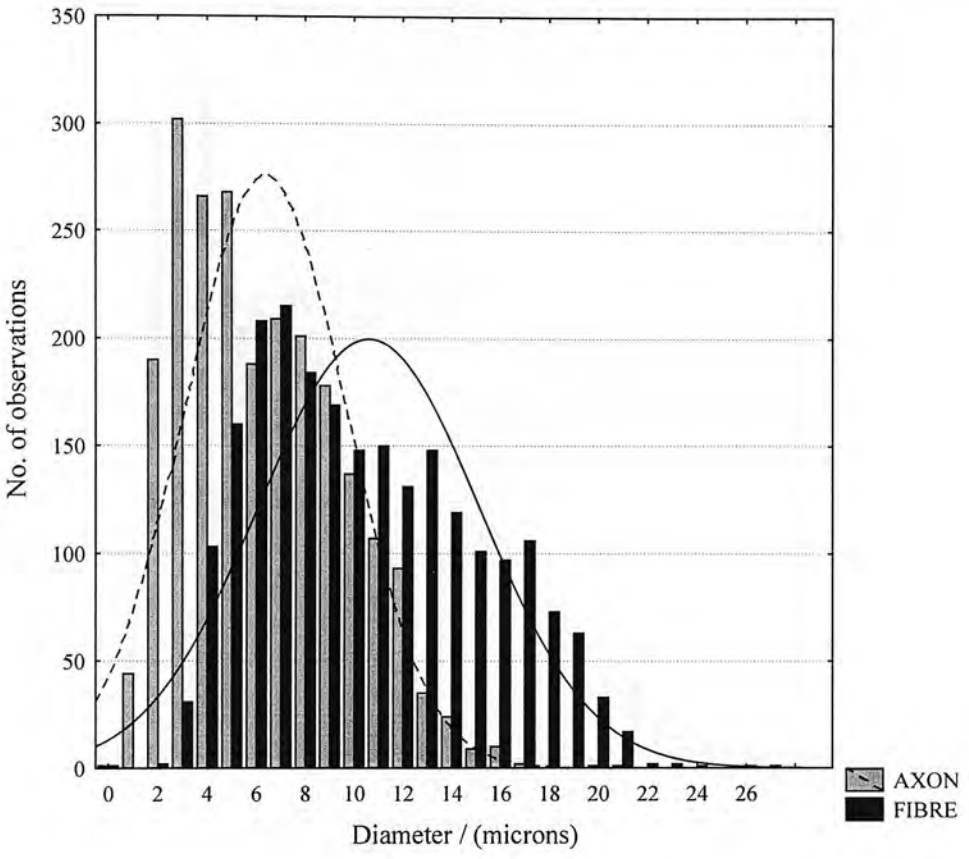


Graph 19 To show the differences in the axon and fibre diameters and the myelin thicknesses of the ulnar nerves distal to the neurorraphy sites for the end-to-side groups compared to normal.

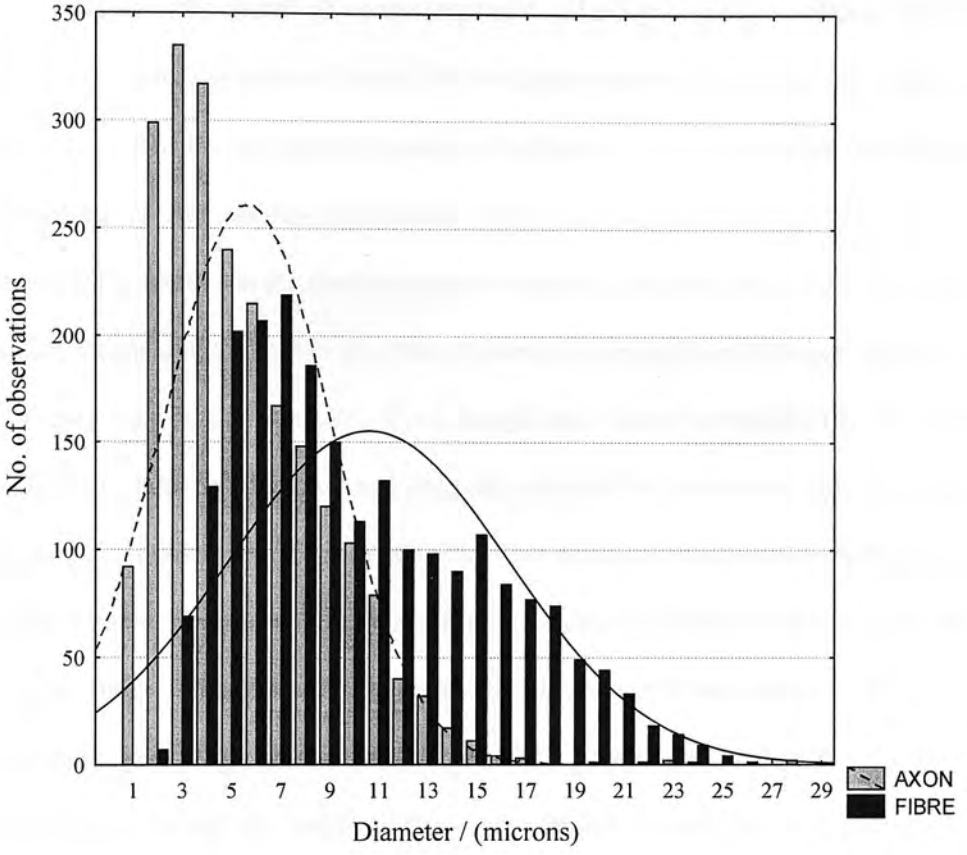
The histograms below show the distribution of the axon and fibre diameters for the normal ulnar nerves and the distal segments of the ulnar nerves of the end-to-side and double end-to-side groups. The bell-shaped curves have been stretched out to the left for both these experimental groups compared to normal but this is not an obvious as the left shift for the median nerves of the these experimental groups. The axon and fibre diameters have both decreased compared to normal, which suggests that there is a degree of damage to the donor ulnar nerve during the neurotomy. There is a larger distribution of the axon and fibre diameters in the experimental groups compared to the normal group again suggesting some fibres have undergone axotomy and regenerated. Regenerated nerve fibres after axotomy are always smaller in diameter than normal nerve fibres. In the median nerve graphs, there is much more of an obvious shift of the whole bell-shape to the left suggesting that all fibres are of similar diameter. These observations are demonstrated in the photomicrographs of the nerve sections.



Graph 20 Histogram to show a normal model distribution of axon and fibre diameters for a normal ulnar nerve



Graph 21 Histogram to show a normal model distribution of axon and fibre diameters for the distal segment of the ulnar nerve of the end-to-side group.



Graph 22 Histogram to show a normal model distribution of axon and fibre diameters for the distal segment of the ulnar nerve of the double end-to-side group.

The myelin sheath thickness appeared to be significantly smaller in the end-to-side compared to normal but not for the double end-to-side group. This gives the impression that the myelin thickness is a lot thicker in the double end-to-side group compared to the end-to-side group. However, the G-ratio was shown to be statistically smaller in the double end-to-side group compared with the other groups, which means that that either the fibre diameter was significantly larger, or the axon diameter significantly smaller. Even though there were no statistically significant differences between the axon and fibre diameters of the two end-to-side groups, the means of the diameters do show that the fibre diameter was slightly higher and the axon diameter slightly smaller in the double end-to-side group compared to the end-to-side group. This theoretically means that the mean values of the myelin sheath thickness were larger in the double end-to-side group compared to the end-to-side group. Even though the results of these experiments showed this, statistically, there was a high probability that there were no significant differences between the means of this variable between the end-to-side groups.

The mean values for the G-ratio were statistically the same in the normal group and end-to-side group meaning that the axon and myelin measurements have decreased in proportion. The axon and fibre diameters of the end-to-side groups have both decreased significantly from normal so the myelin sheath thickness cannot be statistically the same in the double end-to-side group compared to the normal group.

It is likely that the slight differences in the means of the axon and fibre diameters for the end-to-side groups make it appear that the myelin thickness is falsely large in the double end-to-side making the G-ratio for this latter group falsely

smaller than those of the end-to-side groups and the normal group. It is more likely then that the results of the ulnar nerve histological studies show that the means of the axon and fibre diameters of the end-to-side groups have both decreased significantly from normal but not from each other. However ther is a wide range of axon and fibre diameters in the donor ulnar nerves with some being of normal size. The mean values of the myelin thicknesses were likely to be the same in the end-to-side groups compared to each other but significantly smaller than the mean values for this variable of the normal group. The G-ratios for the three groups are also likely to be similar to each other meaning that the nerve fibres are probably mature. This reasoning corresponds well with the findings of the 'jitter' experiments for the donor ulnar nerves.

SUMMARY OF RESULTS

Conventional repair groups

- The fibre diameter and myelin sheath thickness was found to be smaller in the graft group compared to the wrap and neurotmesis groups. The axon diameter and G-ratio, however, was similar in all 3 groups.
- The mean peak tetanic force was found to be larger in the wrap group compared to neurotmesis group. However a more accurate measurement of the tetanus is the peak forces per unit weight of the FCR muscle. The mean values of this variable do not show any significant variance between the groups.

End-to-side repair groups compared with conventional repair and normal groups

- The mean values for the 'jitter' are significantly larger in the end-to-side group compared to the double end-to-side, conventional repair and normal group.
- The mean values of the 'jitter' for the normal group are significantly smaller than those of all the experimental groups. The mean 'jitter' values of the double end-to-side group and the conventional repair group are not different from each other.
- The mean values of the CV_{max} for the normal median group are significantly larger than those for all the other (experimental) groups.

- The mean values of the CV_{max} for the conventional repair group are significantly larger than those of both the end-to-side groups and the 'bridge' group.

- The mean values of the CV_{max} for the end-to-side pathways of the end-to-side group compared to the double end-to-side group are not significantly different. Nor are the mean values of the CV_{max} for the end-to-side group compared to those of the 'bridge' pathway significantly different.

- The mean values of the mass and weights of the FCR muscles of the control group are significantly larger than those of the experimental groups. There are no significant differences in the masses or weights of the FCR muscles between the experimental groups.

- The mean values of the masses of the FCU muscles of the end-to-side group are significantly smaller than those of the double end-to-side group and the normal group.

- The mean values of the myelin thickness and the axon and fibre diameters for the part of the median nerve distal to the repair site for the normal group of animals are significantly different from all the experimental groups. These variables for the conventional repair group are significantly larger than those for both the end-to-side groups. The mean values of the fibre diameter and the myelin thickness of the end-to-side group compared to the double end-to-side group are not different from one another but the axon diameter is larger in the end-to-side group.

- The mean values of the axon and fibre diameters for the ulnar nerves distal to the neurorraphy sites of both the end-to-side groups are significantly smaller than those of normal.

- The mean values of the myelin thickness of the end-to-side group are significantly smaller than those of the normal group, but for the double end-to-side group, the myelin thickness values are not different from normal.

- The mean values of the G-ratio for the ulnar nerves distal to the site of repair are significantly smaller in the double end-to-side group compared to normal. These values are also significantly smaller for the double end-to-side group compared to the end-to-side group. However the values for these variables are not different in the end-to-side group and the normal group.

CHAPTER 4 - DISCUSSION

THE CONVENTIONAL REPAIRS

The results of the experimental tests used to assess the different types of nerve repair of the conventional repair groups of animals did not show any real differences for any of these tests.

End-to-end primary nerve suture is seen as the gold-standard of nerve repair but in general, nerves regenerate poorly. Vast amounts of experimental work over the last 20 years have been carried out using different types of grafting and conduit techniques. Although some improvements in the outcome of nerve repair have been made, opinion on the validity of different types of nerve repair still remains divided.

The conventional repair cohort of sheep consisted of three groups of sheep that underwent procedures that have been shown clinically to support nerve regeneration. The first of these groups was the neurotmesis or primary end-to-end epineurial suture group. This method of repair has long been accepted as the 'gold-standard' if it can be performed without tension on the nerve and in an infection-free environment (Birch, Bonney, & Wynn-Parry 1998a; Sunderland 1978b). Delayed nerve repair i.e. that performed after the first 3 to 4 days after injury has now been shown in clinical and animal studies to be inferior to primary nerve repair (Birch & Raji 1991; Bolesta et al. 1988; Glasby, Fullarton, & Lawson 1997; Glasby, Fullarton, & Lawson 1998).

The second group was that of primary nerve grafting using an autologous 1cm length of nerve of similar calibre to the median nerve being repaired. Despite early reports that nerve grafting was much inferior to end-to-end suture (Smith 1966;

Sunderland 1978c) much work has gone into addressing and attempting to solve the problems often associated with it. These include the limit of the length of graft that can support nerve regeneration, the longer the graft, the increased likelihood of axons not finding their way towards the distal portion of the nerve. Regenerating axons use the endoneurial tubes of the graft as a basement membrane or conduit to grow along but the fasicular pattern of the nerve is complicated and the fascicles tend to branch several times along the length of the nerve or graft trunk (Sunderland 1968). Regenerating axons can become 'lost'. In order to function well the nerve graft has to survive. After transplantation spontaneous revascularization occurs so it is favourable for the recipient site to be rich in blood vessels. Pedicled and free nerve grafts where the nerve graft is harvested with its blood supply still attached or re-attached at the grafting site, have been used experimentally and clinically with some good results, but these were no better than those of a free nerve graft in terms of the functional capacity of the limb (Taylor & Ham1976; Townsend & Taylor 1984). The thicker the nerve graft the longer it takes for the central part of the nerve to become revascularized. If the blood supply is not established by the third day the graft can become fibrotic and disturb the endoneurial tube pattern and damaging Schwann cells (Millesi 2000).

Pioneering work by Millesi (Millesi 1973; Millesi 1981; Millesi, Meissl, & Berger 1972) has improved the outcome of function of a limb by ensuring the individual fascicles of the nerve graft and the nerve to be repaired are aligned and sutured separately. This was best achieved by using the interfascicular graft technique. The results in terms of function of a limb have shown in some studies that an interfascicular nerve graft can achieve results as good as those achieved by end-

to-end repair, however, reinnervation of the muscles supplied by the repaired nerves was quicker in an end-to-end repair than in an autologous nerve graft (Bratton et al. 1979; Tupper, Crick, & Matteck 1988; Young, Wray, & Weeks 1980). Studies have shown that tension should be avoided at the sites of neurorraphy (Daoutis et al. 1994; Driscoll, Lawson, & Glasby 2002; Hoen & Brackett 1955; Inserra et al. 2000). Millesi showed that axon sprouts are able to cross two sites of repair more easily than one site under unfavourable conditions such as a dirty of infected wound (Millesi 1977). However, Sunderland (Sunderland 1990) takes the opposite view that crossing two suture lines is more detrimental than tension on the nerve during repair owing to increased problems with fibrotic tissue and axons making connections with appropriate endoneurial tubes to grow down. Despite this difference in opinion, where a deficit in the nerve exists, repair is best undertaken with a nerve graft.

Nerve grafting is a reliable technique for peripheral nerve repair but is associated with donor nerve deficit. Many other types of nerve conduit have been tried in experimental and clinical studies in the last few decades, using autogenous and exogenous materials. More recently artificial and biodegradable materials have been used. Vascular conduits such as arterial and venous tissue have been shown to support nerve regeneration well, with return of acceptable limb function in rats. They work well when the nerve gap is short (4-15mm) (Benito-Ruiz et al. 1994; Chiu, Janecka, & Krizek 1982; Wang et al. 1993). These studies were carried out by inserting the two transected nerve ends a few millimetres into the vessels and suturing them in place. The size of the nerve gap could therefore be adjusted. Wang et al compared vein grafts with conventional nerve grafting and found that the results in terms of conduction velocity and axon counts were superior in the vein graft group

(Wang et al. 1995). Fascia (Watanabe et al. 2001), collagen (Colin & Donoff 1984; Eppley & Delfino 1988) and muscle (Lawson & Glasby 1998) have also been shown to support nerve regeneration well.

Studies performed with non-biological artificial conduits have showed equally favourable results in terms of nerve regeneration and return of limb function in several experimental studies. Lundborg undertook a prospective randomized clinical study where silicone tubes were used to repair 3-4mm defects in the ulnar and median nerves of the human forearm. No differences were found between the tube and the nerve grafts in terms of function of the limb (Lundborg et al.1994b).

Non-degradable nerve guides have the relative disadvantage that they remain *in situ* as foreign bodies, and can cause chronic foreign body reactions with excessive scar tissue formation and compression of the nerve. They often had to be removed at a second operation (Dellon 1994). Biodegradable substances have been shown to be superior in this respect (Mackinnon & Dellon 1990b). Biodegradable glass is a promising material in terms of its ability to support nerve regeneration and its inertness and degradability. It has been used in a few studies, the present work included, either as a tube or a fabric, with good evidence of nerve regeneration and return of muscle function (Gilchrist et al. 1998).

The work discussed here shows that nerve repair can be performed in many different ways and with some good evidence that these repairs can be similar in terms of return of function to a limb. The results of the repair groups tested here in this work support this finding. Because there were no differences between the groups, it was felt that a 'conventional repair' group consisting of the wrap, graft and end-to-end suture repairs as a single cohort rather than separate groups should be

used for comparison with the end-to-side repairs and the normal group. This made presentation and interpretation of results clearer and more relevant to this study.

It is known that the fibre diameter and myelin thickness decrease after a nerve has been repaired and the maximum conduction velocity is always reduced. The results of the work fit in with these facts. The jitter values were significantly higher for the repairs than for normal FCR muscles, which would suggest that reinnervation of the muscles was still not complete, however there is a degree of instability of the membrane potentials at the motor end plate after nerve repair and this may never return to normal (Kimura 1989a). There is usually a deficit in peak force of contraction of the muscle after nerve repair (Gutmann & Young 1944; Sunderland 1978) but this has not been found in the present experiments. It is unlikely that this result was due to superior nerve repair than in previous studies. It is possible that the calibration of the transducer was not sensitive to measure small changes in force between the conventionally repaired muscles and normal muscles. More probably, small differences would have been recorded with larger groups of animals. However such small differences would be of little or no clinical significance. What is important to note is that the results of the different methods of repair and their inferiority to normal nerves is exactly the pattern of recovery that would be expected from other laboratory and clinical studies. This therefore validates the 'conventional repair' cohort as a legitimate standard against which the conventional end-to-side techniques may be compared.

THE END-TO-SIDE GROUPS

Histology

After nerve repair it is known that the fibre diameter and the myelin sheath thickness decrease. The reasons for this are not fully understood but it is known that the endoneurial tubes of the nerve shrink during Wallerian degeneration and fibrosis prevents re-expansion of the tube to its former size (Gutmann & Sanders 1943; Sunderland & Bradley 1950). During nerve regeneration, unmyelinated axon sprouts develop and elongate either from the nodes of Ranvier (collateral sprouts) or from the cut ends of the nerve axons (terminal sprouts). Each axon in the proximal stump could contribute up to 6 to 20 sprouts. This explains the large increase in fibre numbers seen after nerve repair (Aitken & Thomas 1962; Bray & Aguayo 1974). Axonal sprouts that do not make peripheral connections with a motor end organ eventually die away and the remaining fibres increase in size during a maturation phase (Bray & Aguayo 1974; Sanders & Young 1944). Decreased fibre diameter in regenerated nerve fibres is another consequence of the increased number of nerve fibres but also depends on the size of the parent fibre. Fibres that connect with inappropriate end-organs are able to gain a myelin sheath but never regain a normal diameter (Sanders & Young 1947). The size of the recipient endoneurial tube is also thought to play a role in the eventual size of the nerve fibre. The myelin sheath thickness increases with time and increasing fibre diameter after nerve repair but never returns to normal (Gattuso et al. 1989).

The results in these experiments have shown that fibre diameter does decrease after nerve repair for all the experimental groups compared to those of the

normal median nerves. The same is true for the myelin thickness. The fibre and axon diameter distribution histograms show convincing evidence that these variables are significantly smaller in the end-to-side groups compared with normal median nerves. The results also show that an end-to side or a double end-to-side or 'bridge' technique of repair is worse in terms of these histological properties than a nerve repair by a conventional methods such as a nerve graft or end-to-end suture. Smaller fibres mean the conduction velocity of the nerve decreases (Sunderland 1978) and impairment of recovery of function. However the difference between an end-to-side and double end-to-side repair in terms of fibre diameter is inconclusive in these experiments. This is because although the results show that the means of the axon diameters were significantly larger in the double end-to-side group compared with the end-to-side group, this is unlikely to be accurate because there were no significant differences between the fibre diameters and the myelin sheath thickness for these two groups. Further experiments would have to be carried out with larger numbers of animals in the groups to produce more accurate results.

The histological results for the ulnar nerves showed that the axon and fibre diameters were significantly smaller than both of the end-to-side groups compared with normal nerves, but with a wider range of fibre sizes. Some donor nerve axons were measured as being similar to those of the normal ulnar nerves. This may indicate that there was injury to some of the axons of the donor ulnar nerves caused during end-to-side neurorraphy. This damage is unlikely to be a neurapractic as the fibre diameters of the nerve would recover to their full size after this type of injury. The injury to the axons may range from an axonotmesis to neurotmesis. The double end-to-side group potentially underwent a more serious injury than the end-to-side

group as a double epineurial window was made in the donor nerves of this group, however the results of the histology for these groups showed inconclusive differences (see results for ulnar nerve histology). It cannot be said therefore that there were any differences between the double end-to-side repair and the end-to-side repair.

On microscopic examination of the slides of the nerve sections it was observed in some of the double end-to-side sections that there were axons growing outwith the epineurium. This supports that regenerating axons from the proximal neurorraphy site may have been using the epineurial layer of the segment of donor nerve between the neurorraphy sites ('bridge') as a conduit for reinnervation of the distal attached median nerve and FCR muscle. Reinnervation of the FCR muscle may also have been by regenerating axons from collateral sprouting from the donor nerve at the distal end-to-side neurorraphy. This can be surmised because the end-to-side conduction velocity studies and the F wave studies showed that the end-to-side pathway transmitted measurable all-or-nothing electrical impulses.

Jitter

Stimulated jitter offers a way of monitoring the progression of nerve regeneration quantitatively (Lenihan 2000). In a motor unit that is regenerating there is a larger variability in the time of impulse transmission ('jitter') owing to the lower and more variable threshold potentials at the end plate (Trontelj, Stålberg, & Mihelin 1990).

The results from the present experiments show that the values for the jitter were significantly smaller in the end-to-side experimental groups compared with the normal group, which may mean that regeneration in the experimental repair groups

was not complete and the motor end plates were not mature or had not regained normal physiological properties in terms of neuromuscular variability. There were no differences between the double end-to-side group and the conventional repair group but these two groups show smaller jitter values than those of the end-to-side group. The end-plates for the end-to-side group are less stable. This result implies that the majority of the end-plates in the double end-to-side group and the conventional repair group were more mature than those of the end-to-side group. This may mean that nerve regeneration in the latter two groups had been more effective or more rapid. In order to assess this further it may be necessary to assess the jitter values of the reinnervated muscles sequentially to look at whether the jitter values are decreasing implying maturation of the end-plates.

It was difficult to measure the jitter in the end-to-side groups. The FCR muscles of these groups were often extremely small and degenerate and difficult to locate especially in the end-to-side group. It was sometimes necessary therefore to perform the jitter test by inserting the needle directly into the muscle after opening the skin surgically. Some of the muscles in the end-to-side group did not contract at all when the muscle was stimulated; this decreases the effective size of this group, which lessens the validity of the results. This was not a problem in the double end-to-side group, all muscles contracted adequately for a value of the jitter to be measured.

The results of the jitter of the FCU muscles show that there were no differences in the values between the two end-to-side repairs and no differences between these two groups (end-to-side and double end-to-side) and normal. This could mean that either the fibres of the donor ulnar nerves were not damaged during

the neurorraphy procedure or that if damaged, regeneration of the ulnar nerves or FCU muscles are complete.

Maximum conduction velocity

The maximum conduction velocity *(CV_{max})*, has for many years been the most reliable method for assessing and monitoring the progress of nerve regeneration (Birch, Bonney, & Wynn-Parry 1998). It has been shown in cats that the CV_{max} only returns to 80% of normal velocity after nerve transection and suture (Berry, Grundfest, & Hinsey 1944). Conduction velocity is also affected by a decrease in fibre diameter and myelin thickness and internodal length (Berry & Hinsey 1946; Waxman 1980), but it is thought that these reductions are not solely responsible for the decrease in conduction velocity seen in repaired nerves (Cragg & Thomas 1964).

These results show a significant reduction in the CV_{max} for the experimental groups compared to normal median nerves.

End-to-side repair

Both the end-to-side and double end-to-side groups involve an ulnar donor nerve to distal recipient median nerve pathway. These repairs were compared with the conventional repair and normal median nerve group. The conventional repair group showed a larger mean CV_{max} than the end-to-side and double end-to-side groups but it was smaller than in the normal group. There were no significant differences in the CV_{max} pathways for the end-to-side and double end-to-side groups although measurement of the CV_{max} for the double end-to-side group was more consistent than in the end-to-side group. This may be because some of the muscles were very degenerate in the end-to-side group, and technically obtaining the

measurement was difficult as there was a lot of interference from contraction of surrounding muscles. The CMAPS obtained from these muscles generally, were extremely small which made measurement difficult and prone to inaccuracy.

Conventional repair techniques, collectively, were superior to end-to-side and double end-to-side repair even if the proximal part of the transected nerve is sutured end-to-side a distance proximal to the neurorraphy of the distal stump of the recipient nerve to the donor nerve *i.e.* double end-to-side repair. With this double neurorraphy method, if the proximal stump of a transected nerve is available but cannot be used to repair the nerve by the usual method of end-to-end suture, a neurorraphy made by suturing this proximal stump to the donor nerve at a point where the stump can lie without tension, may act as a conditioning lesion and provide an accumulation of neurotrophic and neurotropic factors that stimulate nerve regeneration. Conditioning lesions have been shown to encourage more rapid nerve regeneration at sites distal to their situation (Bisby 1985; Bisby & Keen 1985; Sjoberg & Kanje 1990). In terms of the CV_{max} for the end-to-side pathways for these results there is no advantage in suturing the proximal stump of the transected nerve to the donor nerve as well as the distal stump.

'Bridge' repair

Another possible route of FCR muscle reinnervation could be along the epineurium of the section of median nerve in between the two neurorraphy sites of the double end-to-side group. Axonal sprouts from the proximal stump of the nerves can use the epineurium or the perineurium of the donor nerve to which it is attached by an end-to-side neurorraphy to act as a conduit to guide growing axons towards the

distal stump. This technique has been tried experimentally in rats and this 'bridge' pathway has been shown to support nerve regeneration (McCallister et al. 2001). The results of CV_{max} measurement for the bridge pathway compared to the end-to-side pathways in the end-to-side group show that there are no differences in the means of the CV_{max} among these three groups. However, the consistency of the results in the double end-to-side and bridge groups is much better than in the end-to-side group which means that these results are likely to be more accurate than those of the end-to-side pathway. The mean CV_{max} of the bridge pathway (28.5 m s^{-1}) are higher than those of the double end-to-side pathway (20.6 m s^{-1}) but are not significant in these experiments (p=0.06). Reinnervation of the FCR muscle in the double end-to-side group could be occurring via two possible pathways simultaneously. Firstly from the end-to-side connection where collateral sprouts from the intact donor nerve were growing into the distal stump and secondly from the bridge pathway, where terminal sprouts from the proximal end-to-side were using the epineurium of the donor nerve as a conduit to guide their growth towards the distal nerve stump. The results show that the effects, in terms of the conduction velocity of the two pathways, are not additive. Two separate sources of growing axons will only make the same number of functional connections with the end organs as one group of growing axons. It may be that there are too many axons at the distal stump and fewer rather than more of them will find endoneurial tubes to continue their growth down.

The mean values of the CV_{max} for the ulnar nerves showed that there were significant differences between the double end-to-side group and normal and end-to-side groups. There were no differences between the end-to-side group and the

normal group. There are several conclusions that can be drawn here. The first is that during the process of making the epineurial window in the donor nerve and end-to-side neurorraphy, there was damage inflicted on the donor nerve. For the end-to-side group this may not have been significant enough to be measured as a difference in conduction velocity. However, because for the double end-to-side group the surgical trauma is approximately double, the damage may be severe enough to make a difference. The type of injury that occurs during the making of the window may be no more severe than a neurapraxia in the end-to-side group as the conduction velocities are similar to those of normal. However the nature of the injury can really only be guessed at for this group and is most likely to be a limited neurotmesis. The type of injury incurred in the double end-to-side group is also likely to result in a partial axonotmesis or partial neurotmesis of the donor nerve. Every care was taken during the experimental process to avoid partial denervation of the ulnar nerve but it practical terms this must be expected to a limited degree. It is important to quantify this deficit in clinical terms but it must be remembered that this technique would only be contemplated after very severe injury.

Muscle physiology

It has been assumed in these experiments that the fascicles exposed after making the epineurial window in the donor ulnar nerve, are all motor rather than sensory fascicles. This was because the neurorrhaphy site was only centimetres from the motor point of the FCR muscle. There were no obvious branches from the median nerve distal to the neurorrhaphy site. However, in the light of this, the fact that nearly half of the end-to-side repairs did not seem to conduct electrical impulses

to produce a motor response does not rule out the possibility that axons regenerated from sensory fascicles to grow into the distal stump. Axons were seen in the sections taken from the attached portion of the median nerve in the end-to-side repairs that did not seem to regenerate, but they were small and probably of dubious functional ability.

The idea of identifying sensory and motor fascicles in order to improve the outcome of nerve repair has been investigated experimentally (Grabb et al. 1970). This involved stimulating the fascicles at the nerve ends and watching for the muscle supplied by that muscle to twitch. If none did that fascicle was classified as sensory. This has been used as a clinical tool by various authors (Haskian 1968, Williams and Terzis 1976), but seems to be of limited value. This method of fascicular screening can not be used before severed axons degenerate, as this requires the patient to be co-operative for sensory fascicle identification and does not differentiate between mixed and sensory fasciculi and mixed and motor fasciculi.

Electrophysiological nerve fascicle identification could be useful in experiments such as these to make sure the distal end of the transected median nerve was being sutured to a motor component of the donor nerve but was out with the scope of this work. However searching for a motor fascicle through a small epineurial window could be technically difficult and may involve making several windows in the donor nerve to find one. This increases the risk of damage to the donor nerve. However this must be considered in the context of the type of injury that is being repaired. It is likely that this technique will be limited to major nerve injuries as a salvage procedure. Such injuries are usually proximal and Sunderland

has shown that the organization of the intraneural plexuses into unimodal fascicles is a phenomenon which increases with distal progression.

The results of the twitch and tetanic tension studies showed that there were no significant differences in the forces produced between the end-to-side groups, the conventional repair groups and the normal muscles.

Most of the FCR muscles in the experimental groups were reinnervated to a certain degree as jitter and conduction velocity values could be measured. It is known from previous work that a muscle only needs 20 % of regenerating nerve axons to make connections with the motor end-plates of the muscle for all its fibres to become innervated and regain near normal force of contraction (Jennekens 1982). This is because regenerating axons tend to develop collateral sprouts, which synapse with neighbouring muscle fibres (increased motor unit innervation ratio) (Rafuse and Gordon 1996). Consequently, muscle fibres of a single motor unit are grouped together instead of being intermingled with fibres of another (Jennekens 1982). Indeed, not all of the regenerating nerve fibres will reach the distal portion of the nerve.

However, force of contraction does not always correlate to function. It is known that after a neurotmesis and subsequent nerve repair humans are always left with some disability (Sunderland 1978). In human experimental subjects, function of a limb is easier to measure objectively than in an experimental animal as voluntary muscle contraction can be used as an assessment tool. We were not able objectively to assess loss of function in the sheep's forelimb, as this was difficult as the FCR muscle only provides a minor role in hoof flexion and therefore it is difficult to know how much this contributes to function of the limb. Under Home Office regulations it

is not ethical to transect the ulnar or radial nerve in the limb as this would affect standing and so the level of functional deficit to the animal is not acceptable (Home Office (H.M.S.O.) 1986). General observation of the animals in the field was a subjective method of assessment and none of the animals was noticed to have any problems with mobility.

The results of the twitch and tetanic tension experiments for the FCU muscles are difficult to draw conclusions from as the number of sheep involved in each group were too small. For future research, it may be that two sheep should be used for each end-to-side nerve repair so that one animal can be used to assess the FCR muscle and the other for assessment of the FCU muscle. However, this would be a doubly expensive experiment.

The area under the curve of the isometric myogram is called the time-tension integral and was originally thought to be a good indicator of the heat produced in the muscle (Hems 1993). The time tension integral actually gives information about the ability of the muscle to move heavy masses rather than heat production. The relationship between the integral and heat production was found not to be proportional as the rate of heat produced in a muscle twitch was not constant (Hartree & Hill 1921). It is thought that a more close relationship between heat produced and mechanical response of the muscle is that between heat production and maximum isometric tension (Fullarton 1994). The time tension index is an indicator of the average tension produced in a muscle. There were found to be no significant differences when the experimental groups were compared with normal animals for any of the twitch tension variables although the mean values for the normal group are nearly twice those of all the experimental groups. The graphs show that the standard

deviation of the values of the peak twitch and time tension index and integral for the groups were wide but less so in the end-to-side group. The number of animals in the end-to-side group though were about half that of the other experimental groups which may make these results less accurate than the other groups.

The time tension integral for the tetanic tensions of the normal FCR muscles had a *p* value of 0.058. The box plot (Graph 11) shows that the standard error of the normal group does not overlap with that of the experimental groups. The mean of this group was 392 mNs whereas the means of the experimental groups lay between 224 and 244 mNs. The mean values of the experimental groups were very similar with a relatively small standard deviation. The size of the normal group compared with the conventional and DE to S group was nearly half however, which perhaps makes the difference between the mean for the normal misleadingly high compared to the experimental groups. After denervation and subsequent nerve repair and reinnervation, it cannot be said that in all cases muscles regained the strength they had before nerve injury (Sunderland 1978).

The integral for the twitch gives an idea of the ability of the muscle to move masses but the integral for a tetanic stimulus may give a better idea of the heat produced in that muscle. The contraction during tetanus is sustained and maximal and is overall a better test of the strength of the muscle. The stimulus is only stopped when the muscle fatigues. Half fatigue is a good reference point from which to measure the integral as it is easy to find this point on the myogram making comparisons of values between muscles more accurate. Even though the mean values of the integral for the tetanus approaches a probability value that might indicate a significant difference between the normal group and the experimental

groups it is difficult to know whether this potentially means anything significant as groups differ in size and the other data for the tetanic tension such as peak tetanic force values and time tension indexes do not show any significant differences.

Muscle mass

When the FCR muscles of the experimental groups were excised it was noted that the muscles of the end-to-side, the double end-to-side and the conventional repair group were generally wasted in appearance compared with normal FCR muscles. This was especially noticeable in the muscles of the end-to-side group. This was not confirmed when the muscles were weighed on a balance and masses (g) compared. The results show that there was no difference in the masses of the FCR between the experimental groups. However the mean masses of the normal muscles were significantly higher than those of the experimental groups.

Even though the masses of the muscles of the experimental groups were significantly smaller than those of the normal groups the values for the twitch and tetanic forces were no different in the experimental and normal groups. The mass of a muscle does not correlate with force of contraction or function of that muscle. Gilmour showed that after nerve transection and repair fast twitch muscles in rats regain only up to 65% of normal muscle mass (Gilmour 1990) whereas Grieve et al (Grieve, Kristmundsdottir, & Glasby 1991) showed a recovery of 85% of the contralateral control fast twitch EDL muscle weight in the adult rat when the transected nerve was repaired by a muscle graft. Fast muscles such as the FCR are permanently affected by nerve injury whereas slow muscles can make a complete

recovery. This may be in part due to the fact that slow muscles are reinnervated sooner than fast muscles (Jaweed, Herbison, & Ditunno 1975).

A study by Berry et al (Berry, Grundfest, & Hinsey 1944) looked at nerve crush versus nerve transection in the sciatic nerves of cats. Almost complete function of the affected limb was restored in nerve crush but in nerve transection and suture, stimulation of the regenerating nerve produced muscle twitches in the gastronemius as early as 23 days but animals did not start to use the limb until two months and never regained normal use. It was thought that the reduction in force produced by the muscle after neurotmesis is due to a decrease in the number of new axons innervating that muscle but in 1988 it was shown that reinnervation of partially denervated hind limbs in cats resulted in an increase in the size of motor units and that full recovery of muscle force could be produced with only 5% of motor axons intact (Sillman et al. 1992). It is difficult to explain the apparent lack of differences in the muscle physiology for the experimental groups compared with normal. The most likely explanation is a combination of small group sizes and 'within groups' variation. The two are additive. Group size in the design of experiments such as this must take into account the statistical probability of achieving adequate statistical power (>0.9 ideally). However, Home Office regulations demand a minimum number of animals to achieve 'an acceptable' result. The cost per sheep of the entire experimental process was about £3,000. A group of twelve sheep therefore requires £36,000 or £18,000 for six sheep. In the light of today's problems with achieving research grants this must be a factor in experimental design. Sheep also vary in size and large sheep possess large nerve fibres. This results in 'within groups' variation which when present may result in a failure of the F test to show differences because

this test relies upon differentiating 'within groups variation' from 'between groups variation'. A more scientific but more costly experimental strategy would be to reduce the value of all measured variables to a value between 0 and 1 by dividing the experimentally observed value by that of the identically measured but otherwise normal observation from the opposite limb (Fullarton, Glasby, & Lawson 1998; Glasby, Fullarton, & Lawson 1997). However, this strategy would greatly increase costs and experimental workload and in no way alters the argument against small groups. The present philosophy must be that although group sizes are less than ideal the advantage of using a large animal model for making clinical predictions so much outweighs the use of rats or rabbits that a less than perfect strategy has to be accepted.

In the double end-to-side group it would also have been interesting, if there had been more time, to measure the muscle tensions of the FCR muscle for the 'bridge' pathway and compare the values of the twitch and tetanic tensions to those of the end-to-side pathway in this group.

F wave

The results of the F wave studies have shown no differences in the minimum latencies between normal median and ulnar nerves and those of the end-to-side groups. The important but only conclusion that can be drawn from this is that there are functioning axons across the end-to-side neurorraphy sites. The axons are conducting electrical impulses and therefore must have been formed from sprouts from the donor ulnar nerve growing into the distal median nerve stump. It was noted also that when the distal part of the attached median nerve was stimulated the corresponding FCU muscle contracted. It was difficult to tell with the naked eye

whether the FCU muscle contracted before the F wave appeared on the Medelec screen. Contraction of the FCU muscle after The F wave is an indication of it being activated by antidromic impulses travelling through true collateral connections with the donor nerve. The presence of F waves on the Medelec screen must signify this pathway conducts electrical impulses through true collateral sprouts. The contraction of the FCU muscle that was observed is more likely to be an M wave from orthodromic activation of the FCR muscle. It would have been interesting, if time for these experiments could have been increased, to try and measure twitch and tetanic tensions of the FCR muscles by stimulating the part of the donor ulnar nerve distal to the end-to-side neurorraphy site and compare these values to those of the twitch and tetanic tensions of the FCR muscle when the proximal part of the ulnar nerve was stimulated (as done in these experiments for the end-to-side groups).

THE USE OF END-TO-SIDE REPAIR AS A CLINICAL TOOL

End-to-side nerve repair has been shown to support nerve regeneration. This is either by terminal sprouting from damaged donor axons injured during the making of the epineurial window or from collateral sprouting from the nodes of Ranvier of the donor nerve. It is to be hoped that the latter process of collateral sprouting occurs rather than terminal sprouting which could compromise the donor nerve and muscles. The efficacy of end-to-side repair has been the subject of wide scientific debate since it's re-emergence in the literature ten years ago. This was mainly due to work by Viterbo (Viterbo, Trindade, Hoshino, & Mazzoni 1992; Viterbo, Trindade, Hoshino, & Mazzoni 1994; Viterbo et al. 1994; Viterbo et al. 1998) and Lundborg (Lundborg, Zhao, Kanje, Danielsen, & Kerns 1994).

The attractiveness of end-to-side neurorraphy for clinical use is in patients who sustain injuries to limbs where large areas of soft tissue are lost. With such injuries nerve grafting can be unfeasible mainly owing to length of the graft that may be needed. Long grafts do not support nerve regeneration well and the amount of donor tissue that may be needed may produce unacceptable further deficit to the patient. In the present day these injuries tend to be sustained from motorbike accidents where victims often suffer severe traction injuries to the brachial plexus. 80% of high velocity gunshot wounds in limbs will cause a serious nerve injury. These injuries cause loss of long lengths of nerve along with iatrogenic situations such as tumour excision. In an age where extreme sports are highly fashionable, an increasing number of fast vehicles are on the roads and conversely, more people are using bicycles to avoid traffic and increase physical fitness, the incidence of such injuries are regrettably increasing. End-to-side repair involves suturing the distal stump of the injured transected to the side of an adjacent intact nerve without tension. If possible it is preferable that the donor nerve does not supply a major muscle group as simultaneous muscle contraction can occur as the donor nerve would then innervate two sets of muscles (Ballance & Ballance 1903). The technique has been used clinically in a few patients with varying results. Some studies show an acceptable return of function (May, Sobol, & Mester 1991)and some, little return of function. It seems to be more useful at the present time in brachial plexus repair, presumably because of close proximity of large nerve trunks that can act as donor nerves. The animal studies on end-to-side repair have almost exclusively involved rats and show that end-to-side is a feasible method of nerve repair in terms of return of function (Tham & Morrison 1998; Tiangco et al. 2001; Viterbo et al. 1994;

Viterbo et al. 1998; Zhang et al. 2000). It was important to further this work by testing the repair on nerves that more closely resemble those of humans to see if the promising results mentioned above were reproducible.

The recipient median nerve and FCR muscle

In this work on the sheep model, the function of two types of end-to-side neurorraphy were tested and compared to conventional repairs and normal median and ulnar nerves.

In the double end-to-side experiments it was hoped that the double neurorraphy of this group would provide an advantage to single end-to-side neurorraphy. An additional effect to the end-to-side neurorrhpahy may have been obtained from regenerating axons from the proximal end-to-side neurorraphy site using the epineurium and perineurium as membranes to guide their growth towards the distal stump of the attached median nerve of the of the distal end-to-side neurorraphy site (McCallister et al.2001).

During epineurotomy it was hoped that axotomy would not occur for fear of damaging the donor nerve but never-the-less this could have happened. It is known that conditioning lesions such as an axonotmesis type injury at a site proximal to a repair site can stimulate and increase the rate of axon growth at the repair site due to activation of neurotrophic and neurotropic substances (McQuarrie & Grafstein 1973; McQuarrie & Grafstein 1977). Transection of a nerve stimulates outgrowth of axons from its cut surface and damaged Schwann cells also act as a stimulus for axon regeneration (Lundborg 2000; Sunderland 1990).

The results of the experiments on the end-to-side groups show that there may be no such advantage offered by a second neurorraphy as the only difference between them for the tests performed on the repaired median nerve and FCR muscles of the groups was for the jitter. The mean values of this were significantly higher in the end-to-side group compared to the double end-to-side group, which could just mean that the muscles have not yet regenerated fully. However, there is little doubt that the results for the jitter, CV_{max}, and twitch and tetanic tensions are much more consistent. Nearly half the FCR muscles in the end-to-side group were so degenerate that they could not be stimulated to twitch even with high stimulus currents. Interestingly, none of the end-to-side neurorraphy sites had dehisced on dissection of the sites at the end of the experiments. This finding could be because theoretically, there are two sources of growing axons in the double end-to-side group and their effects could be additive. However, more axons at the distal neurorraphy site do not necessarily mean more chance of end organ reinnervation as they could become tangled. Also there are only a finite number of endoneurial tubes in the distal median nerve stump for regenerating axons to grow down. Axons could grow on the outside of the attached recipient median nerve trunk using the epineurial sheath as a conduit and neurotize the distal muscle directly (Millesi 2000). Compared to the 'bridge' distance however, the average length of the median nerve stump was 10 times that length. The size of the 'bridge' (distance between the two neurorraphy sites) is small in these experiments. It is known that long nerve grafts do support regeneration but the results in terms of function become less good the longer the nerve graft. Some of the photo micro graphs of the nerve sections of the end-to-side groups show nerve axons in the perineurium of the attached recipient nerve but none in the epineurium

that were easily identified. In practical terms and the clinically relevant point for these experiments is that sometimes the proximal stump of the injured nerve is easily identified and sometimes available and relatively uninjured such as in tumour removal. Even though the proximal neurorraphy site would probably be some distance from the distal site due to nerve loss from tumour involvement or extensive injury and as a result of debridement, this technique may allow the additive affect of the donor 'bridge' to the end-to-side neurorraphy to reinnervate target muscles. The technique acts as an alternative to nerve grafting and works in these experiments, but it would be useful one to further the work and place the proximal neurorraphy site at a larger distance from the distal site.

The contribution of each source of axons to the reinnervation of the muscle cannot be quantified as this is out with the capabilities of these experiments. It would be interesting to find out and may be an area for further study.

These results do therefore shown that a double end-to-side neurorraphy can offer an advantage in regeneration of an attached nerve as it works more consistently than a single neurorraphy. This likely to be because regenerating axons use the bridge as a conduit as well as growing down the end-to-side pathway.

In terms of quality of the repair, the results of both end-to-side techniques are similar to the conventional repairs for some of the tests but not others, which are discussed in the 'conventional repair' section of this chapter. The conduction velocity of a nerve is the best measures of functional outcome of the muscle it supplies and this is significantly better in the conventional repairs than the end-to-side repairs.

The results of the FCR muscle twitch and tetanic tensions for the end-to-side repairs did not show significant differences from normal or the conventional repairs, nor was there any difference between the end-to-side groups. However the sheep were age-matched but not matched for weight. It was difficult at the time these experiments were taking place to find sheep of similar weight and size as there was a sudden shortage of these animals that could not resolved. Some of the measurements of the muscle forces could be expressed per unit weight of the muscle, which helped to overcome this problem. It was probably fair to expect that the values for the twitch and tetanic forces would be smaller in the experimental groups compared to normal as this has been shown in previous studies on muscle function after nerve repair (Birch, Bonney, & Wynn-Parry 1998; Sunderland 1978). The twitch and tetanic tension values were similar in the end-to-side groups and the conventional repair, which perhaps shows that even though the electrophysiological results may be superior for a conventional repair, muscle strength is similar to an end-to-side repair. This however cannot correlate to function.

Effects on the donor ulnar nerve and FCU muscle

It can be argued that if terminal sprouting occurs from donor nerve axons that have undergone axotomy during epineurotomy, these are lost to the donor nerve and may compromise its ability to conduct nerve impulses normally (Viterbo, Trindade, Hoshino, & Mazzoni1992). End-to-side neurorraphy with deliberate axotomy has been shown in several clinical studies to reanimate facial muscles (May, Sobol, & Mester 1991) and in brachial plexus repair to work well (Nath & Mackinnon 2000). Regenerating proximal sprouts may enter either the endoneurial sheaths of the distal

donor nerve, or the endoneurial sheaths of the recipient nerve. Motor unit re-modelling in the distal part of the intact donor nerve motor end organs compensates for the loss of motor axons that become diverted through the end-to-side graft. This re-modelling may reduce or eliminate any donor nerve functional deficit (Foehring, Sypert, & Munson 1986). However there have been other animal studies where axotomy has not been deliberate and nerve regeneration has been supported with good return of function to the limbs involved with 60-90% of normal force regained (Cao, Shidao, & Yu 1997; Kalliainen, Cederna, & Kuzon, Jr. 1999; McCallister, Tang, & Trumble 1999). However it is difficult objectively to measure the amount if any, of damage actually being done the donor nerve during epineurotomy in practical terms.

This work has shown that differences in the function of the donor FCU muscles and ulnar nerves lie in the CV_{max} and the mass of the FCU muscle. The mean value of the conduction velocity of the double end-to-side group was significantly smaller than those of the end-to-side group, which were similar in the latter two groups. It could be that the double neurorraphy had caused axotomy of some of the donor nerve fibres promoting terminal sprouting rather than collateral sprouting to the end-to-side pathway with loss of these axons to the donor nerve.

The mass of the FCU muscle of the end-to-side group is significantly smaller than those of the double end-to-side group and the normal group. These latter two groups have FCU muscles that are similar in mass. This is most likely due to error in removing the muscle from the animal as the other tests performed on the ulnar nerves and muscles of this group do not show any differences from normal or the double end-to-side group. The reason for the error could be in inconsistencies in the amount

of muscle being removed. The FCU muscle was a large bipennate muscle, closely associated with neighbouring musculature and therefore difficult to define during dissection. The end-to-side experiments were performed first followed by the double end-to-side and then the normal FCU muscles. It is likely the consistency in removing the same amount of muscle for each animal improved as the number of removal procedures increased. This could be improved by practising the procedure on non-experimental animals prior to the experimental animals. However this would be costly and and may not concur with Home Office regulations.

This experimental work has shown that an end-to-side neurorraphy on its own supports nerve regeneration but this is unpredictable. Therefore at this time, this technique cannot be recommended for widespread clinical use. When regeneration does occur successfully, good muscle function can be restored. The addition of proximal stump suture increases this predictably to a clinically acceptable level, if the bridge distance is short, as the results of return of function to muscle compare similarly with clinically acceptable conventional methods of nerve repair. Even though in the present time there could be a large demand for such a technique, these results have shown that it should only be used as a last resort form of management

CONCLUSIONS

The following conclusions can be drawn from this work:

In terms of the electrophysiological, morphometric and muscle physiology tests performed on the all the nerve repairs after nerve transection from the groups of animals:

- There were no significant differences in the outcome of a nerve repair using a biodegradable glass entubulation technique, an autologous nerve graft or direct end-to-end epineurial suture.
- End-to-side nerve repair did support nerve regeneration but was all or nothing. When reinnervation of the distal nerve stump and the recipient FCR muscle did occur, the functional outcomes were inferior to conventional techniques of nerve repair apart from the twitch and tetanic tensions of the FCR muscle.
- Double end-to-side nerve repair consistently supported nerve regeneration but this repair was inferior to conventional techniques of nerve repair in all measures of outcome except twitch and tetanic muscle tensions. It is likely that regenerating axons used the epineurial and perineurial layers of the donor nerve segment between the two neurorraphy sites as a conduit for axon growth as well as regenerating axons from collateral sprouts in the end-to-side pathway.
- The function of the donor ulnar nerves in terms of conduction velocity, were compromised in the double end-to-side repair but not the end-to-side repair. Assessment of the donor FCU muscles in terms of the muscle physiological experiments also needs to be properly tested.

- Further study is needed to assess the effects of placing the proximal neurorraphy site further away from the distal site in a double end-to-side neurorraphy on nerve regeneration. It is not clear from this work why some of the end-to-side neurorrhaphies supported nerve regeneration and some did not, especially in the light of the fact than none of the repairs had dehisced macroscopically.
- The present findings while confirming that nerve regeneration can take place through end-to-side nerve repair do not support the view that this technique is associated with a functional outcome, which would justify its use in clinical practice.

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APPENDIX

SOLUTIONS USED FOR PROCESSING OF NERVE TISSUE FOR HISTOLOGICAL EXAMINATION

0.4M Sodium Cacodylate – 8.561g sodium cacodylate was dissolved in 50ml of distilled water and then made up to 100ml with more distilled water.

0.2M Sodium Cacodylate Buffer (pH=7.4) – 8mls of 0.2M hydrochloric acid was added to 50ml 0.4M sodium cacodylate until the pH=7.4. This was then made up to 100ml with distilled water.

10% Sucrose in 0.1M Sodium Cacodylate Buffer – 10g of sucrose was added to 50ml of 0.2M sodium cacodylate buffer and dissolved. The pH was then corrected to 7.4 and the volume made up to 100ml with distilled water.

2.5% gluteraldehyde in 0.1M sodium cacodylate buffer - 50 ml of 0.2M sodium cacodylate buffer, pH 7.4 was added to10 ml of 25% gluteraldehyde. This was made up to 100 ml with distilled water.

5% sucrose buffer solution - 50 ml of 0.2M sodium cacodylate buffer was added to 5.000 g of sucrose using a magnetic stirrer. The pH was 7.4 and the solution was made up to 100 ml with distilled water.

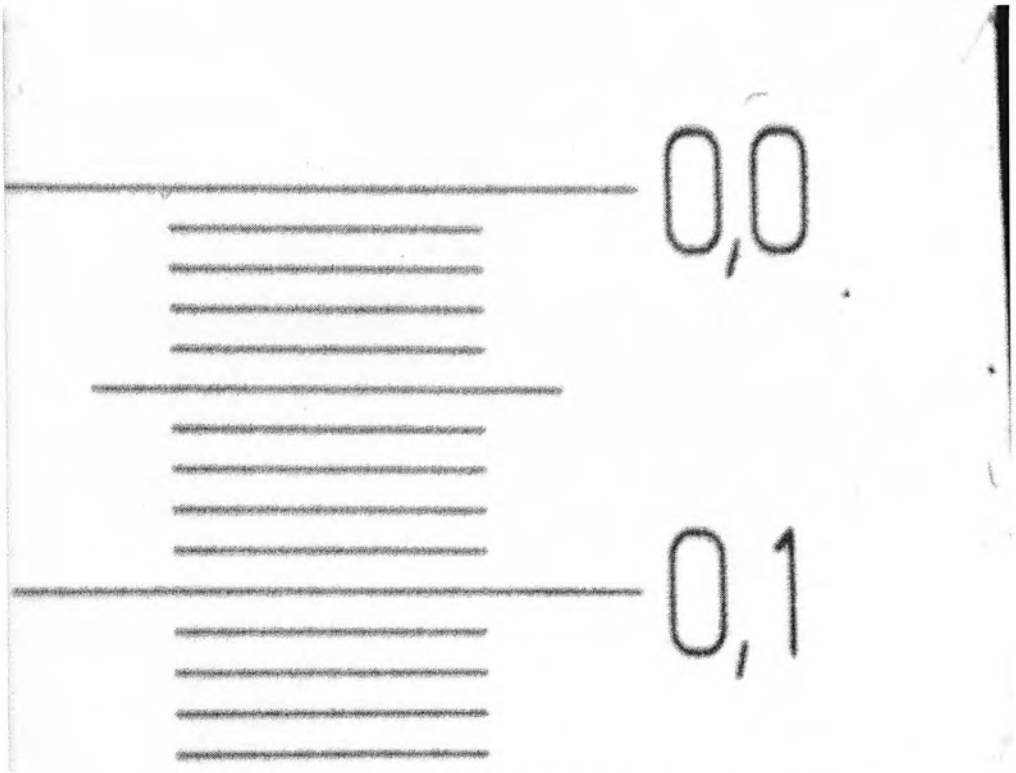
1% Osmium Tetroxide in 0.1M Sodium Cacodylate Buffer – Osmium tetroxide is dangerous and was only handled in a fume cupboard. A 0.1 g ampoule of osmium tetroxide with its paper label removed was cleaned with absolute alcohol to remove all trace of the paper. This was placed in a universal bottle, with the cap tightly screwed closed, and shaken to break the ampoule. 5 ml of 0.2M sodium cacodylate buffer, pH 7.4 and 5 ml of distilled water was added and shaken to mix. This was placed overnight in the refrigerator to allow the crystals to dissolve.

Araldite epoxy resin - Araldite causes skin irritations and was handled in a fume cupboard. There were four components of the resin which when mixed slowly polymerise:

1. Araldite CY212
2. Dodecenyl succinic anhydrite (hardener)
3. Dibutylphthalate (plasticizer)
4. Benzyldimethylamine (accelerator)

1 and 2 were mixed in equal proportions. 3 and 4 were mixed in a 2:1 mix (two measures of dibutylphthalate with one measure of benzyldimethylamine). A universal bottle was filled to its 'shoulder' with the 1 + 2 mixture and 0.5 ml of the 3 + 4 mixture was added. This was left overnight on the rotator and store in the refrigerator.

Pyronin B and Toluidine Blue stain - 1 g of di-Sodium tetrahydoborate decahydrate (Borax) was mixed in 100 ml of distilled water (1% solution) with a magnetic stirrer for 1 hour). 1 g of toluidine blue ($C_{15}H_{16}N_3SCl$) was added and again stirred for 1 hour (this makes 1% solution of toluidine blue in 1% borax). 1 g of Pyronin B was mixed in 100 ml of distilled water (1% solution, use magnetic stirrer for 1 hour). Four parts of the 1% solution of toluidine blue was mixed in 1% borax with one part of the 1% Pyronin B solution. The combined solution was filtered to remove any precipitate.



Photomicrograph of a portion of the calibration grid (objective x40) used to calibrate the AIS system used for morphometry.